	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments	Error Definition	Errors
1	BRS	L1	4710	cerbb2 or c-erbb2 or cerbb-2 or c-erbb-2 or her2 or her-2 or p185 or neu	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/02/25 11:04			0
2	BRS	L2	12054 7	antibod$3	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/02/25 11:04			0
3	BRS	L3	483	1 with 2	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/02/25 11:24			0

02/25/2002, EAST Version: 1.03.0002

	Type	L #	Hits	Search Text	DBs	Time Stamp	Comments	Error Definition	Errors
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5	BRS	L5	31	3 with 4	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2002/02/25 11:26			0

02/25/2002, EAST Version: 1.03.0002

(FILE 'HOME' ENTERED AT 10:53:59 ON 25 FEB 2002)

FILE 'MEDLINE, EMBASE, BIOSIS, CAPLUS, CANCERLIT, SCISEARCH' ENTERED AT
10:54:23 ON 25 FEB 2002

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L1      33549 S CERBB2 OR C-ERBB2 OR CERBB-2 OR C-ERBB-2 OR HER2 OR HER-2
OR
L2    2427006 S ANTIBOD###
L3       4126 S L1 (10A)L2
L4       9591 S BISPECIFIC OR MULTISPECIFIC OR BI-SPECIFIC OR MULTI-SPECIFIC
L5        242 S L3 (30A) L4
L6        104 DUP REM L5 (138 DUPLICATES REMOVED)
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    15:45:36 ON 23 FEB 2002
L1   134 S 452F2 OR 741F8 OR 759E3 OR 113F1 OR 454C11
L2   38 DUP REM L1 (96 DUPLICATES REMOVED)

Type	L #	Hits	Search Text	DBs	Time Stamp	Comments	Error Definition	Errors
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1								

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Please send me the following references ASAP:

Journal of Experimental Medicine 175:217-225 (Jan. 1, 1992)

Int. J. Cancer 50:800-804 (1992)

Int. J. Cancer, Suppl. (1992), 7(Bispecific Antibodies Targeted Cell Cytotoxic.), 42-4

PROC AM ASSOC CANCER RES ANNU MEET. (1992) 33 (0), 344

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Cancer Immunol. Immunother. (1994), 39(1), 41-8

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Thanks,

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Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the HER2 Protooncogene

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Summary

The HER2 protooncogene encodes a 185-kD transmembrane phosphoglycoprotein, human epidermal growth factor receptor 2 (p185^HER2), whose amplified expression on the cell surface can lead to malignant transformation. Overexpression of HER2/p185^HER2 is strongly correlated with progression of human ovarian and breast carcinomas. Recent studies have shown that human T cells can be targeted with bispecific antibody to react against human tumor cells in vitro. We have developed a bispecific F(ab')2 antibody molecule consisting of a humanized arm with a specificity to p185^HER2 linked to another arm derived from a murine anti-CD3 monoclonal antibody that we have cloned from UCHT1 hybridoma. The antigen-binding loops for the anti-CD3 were installed in the context of human variable region framework residues, thus forming a fully humanized BsF(ab')2 fragment. Additional variants were produced by replacement of amino acid residues located in light chain complementarity determining region 2 and heavy chain framework region 3 of the humanized anti-CD3 arm. Flow cytometry analysis showed that the bispecific F(ab')2 molecules can bind specifically to cells overexpressing p185^HER2 and to normal human peripheral blood mononuclear cells bearing the CD3 surface marker. In additional experiments, the presence of bispecific F(ab')2 caused up to fourfold enhancement in the cytotoxic activities of human T cells against tumor cells overexpressing p185^HER2 as determined by a ^{51}Cr release assay. These bispecific molecules have a potential use as therapeutic agents for the treatment of cancer.

Recent studies have revealed an association between overexpression of the HER2 protooncogene and the progression of breast and ovarian carcinomas accompanied by worsened clinical outcome (1-3). HER2 encodes a transmembrane phosphoglycoprotein receptor tyrosine kinase with an approximate molecular weight of 185,000 (p185^HER2) whose amplified expression can lead to malignant transformation as determined in soft agar assays and in nude mice model. (4, 5). Thus, HER2 may play a crucial role in the tumorigenesis of breast and ovarian carcinomas in humans (2). Of relevance, cells overexpressing HER2/p185^HER2 exhibit more resistance to cytotoxic effects of monocytes and TNF-α, a cytokine that has direct antitumor activities and is thought to mediate immune cell killing of tumor cells (5).

Bispecific mAbs (BsmAbs) with dual specificities for tumor-associated antigens on tumor cells and for surface markers on immune effector cells have been described (6, 7). These BsmAbs have been shown to be effective in directing and triggering effector cells to kill tumor cell targets (8). One approach to produce BsmAb involves the fusion of two mAb-producing hybridomas to form quadromas that secrete BsmAb in addition to undesirable chain combinations including parental mAbs. Another approach utilizes directed chemical coupling of Fab' fragments from two different mAbs to assemble a BsmAb with the desired specificities (9). Limitations associated with such approaches include the ability of rodent-derived BsmAb to elicit immune response in humans. To this end, genetic engineering techniques have been applied to production of less immunogenic "humanized" antibodies (10, 11). Recently, we have described the humanization of murine mAb 4D5 (mumAb4D5), which is directed against the extracellular domain (ECD) of p185^HER2. The

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[1] Abbreviations used in this paper: BsmAb, bispecific mAb; FR, framework region; hu, humanized; p185^HER2, human epidermal growth factor receptor 2.

humanized (hu) antibody, humAb4D5-8, consists of the antigen-binding hypervariable regions from the murine parent mAb together with human variable region framework residues and constant domains (12).

In this report, we describe the production of humanized BsF(ab')₂ with specificities toward the extracellular domain of p185^{HER2}, and the human T cell surface marker CD3. Thus, one arm is humAb4D5-8 and the other a humanized version of murine anti-CD3 mAb, UCHT1. Our approach involved separate *Escherichia coli* secretion of each Fab' followed by directed chemical coupling reaction in vitro to form the BsF(ab')₂ fragment. Data are presented demonstrating the biological properties of these BsF(ab')₂ molecules, including the specific binding to cells overexpressing p185^{HER2} and to normal human T cells; and their ability to trigger the lytic activity of human CTL against breast tumor targets.

Materials and Methods

Cloning of Anti-CD3 Variable Region Genes. The mumAb anti-CD3-producing hybridoma UCHT1 (13) was used for extraction of mRNA (14). The genes encoding mumAb anti-CD3 variable domains were isolated by PCR amplification of mRNA as described (15). NH₂-terminal sequencing of mumAb anti-CD3 light and heavy chains was used to design the sense strand PCR primers, whereas the antisense PCR primers were based upon consensus sequences of murine FR residues (16) incorporating unique restriction sites for directional cloning shown by underlining and listed after the sequences: V$_L$ sense, 5' TTTAACGCGTACGCT-GACATCCAGATGACCCAGACCACCTCCTCCCTGTCTGC-CTCCCTGGGCGAY3', *Mlu*I; V$_L$ antisense, 5' TTTGCATGCGT-CTTTGGGGTAGAAGTTGTTCAAGAAGCA 3', *Sph*I; V$_H$ sense, 5' AACGCGTACGCTGARGTSCARCTSCARCARTC 3', *Mlu*I and V$_H$ antisense 5' GGCAGAGATCCAGGGGCCCGTGGATA-GACAGATGG 3', *Apa*I; where Y = T or C, R = A or G, and S = C. For each variable domain, the products from two independent PCR reactions were cloned into the pUC119-based phagemid pAK2 (12), and a total of at least five clones was sequenced by the dideoxy method (17).

Molecular Modeling and Construction of Humanized mAb Anti-CD3 Genes. Humanization of mumAb anti-CD3 by installing CDR residues from this murine antibody into the context of consensus human FR sequences was performed as previously described for mumAb4D5-8 (12). Briefly, mumAb anti-CD3 was humanized by judicious recruitment of corresponding CDR residues and a few FR residues into the humAb4D5-8 molecule. Differences between mumAb anti-CD3 and the human consensus FR residues (see Fig. 1) were individually modeled to investigate their possible influence on CDR conformation and/or binding to CD3. Genes encoding humAb anti-CD3 variant 1 V$_L$ and V$_H$ domains were assembled by gene conversion mutagenesis of corresponding humAb4D5 gene segments cloned in pUC119 (12) using 246-mer and 283-mer preassembled oligonucleotides, respectively. Briefly, sets of four contiguous oligonucleotides were designed to create humAb anti-CD3 V$_H$ and V$_L$ utilizing codons commonly found in highly expressed *E. coli* genes (see Fig. 1). These oligonucleotides are 54–85 residues in length, contain 7–17 mismatches to the humAb4D5 template, and are constrained to have eight or nine perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of oligonucleotides were phosphorylated, annealed to corresponding templates, and ligated. Full-length

oligomers were isolated after electrophoresis on a 6% acrylamide sequencing gel and then used for an efficient mutagenesis procedure (12). Clones precisely encoding mumAb anti-CD3 V$_H$ and V$_L$ (Fig. 1) were identified by nucleotide sequencing (17).

Additional humAb anti-CD3 variants were designed in which one or more mumAb anti-CD3 residues were replaced by their human counterparts in order to test their role in antigen binding, namely V$_H$ K73D and/or V$_L$ R53S (a single letter code for the original amino acid followed by the residue number according to Kabbat et al. [16] and then the amino acid replacement). The murine V$_H$ residue, K73, is located in a loop that is close to heavy chain CDRs H1 and H2 (Fig. 2), and might be involved in antigen binding. In contrast, our humAbs usually contain an aspartate at this position as they are derived from a consensus sequence of V$_H$ group III, which is the most abundant human group in the compilation of Kabat et al. (16). V$_L$ residue 53 is an arginine in mumAb-antiCD3 that is located towards the COOH terminus of CDR L2 and may be able to reach up and assist in antigen binding while also interacting with the phenyl ring of V$_L$ Y50 (see Fig. 2). In our humAbs, this residue is normally a serine derived from a consensus sequence of V$_L$ κ subgroup I, which is the most abundant human light chain subgroup (16). These additional variants were generated by an efficient site-directed mutagenesis method (18) using the oligonucleotides:

V$_L$ R53S
5' CTATACCTCCAGC<u>CTCGAG</u>TCTAGGAG 3'
            XhoI

V$_H$ K73D
5' AAGCGTAGATGACTCCAAAAACACAGCCTACCTGCAAAT
<u>GAATTC</u>TCTGCGTGCTG 3'
EcoRI

where an asterisk indicates a mismatch and unique restriction sites introduced are underlined.

Fab' Fragment Expression for humAb4D5-8 and Anti-CD3 mAb Variants. We previously described the vector, pAK19, for the cosecretion of humAb4D5 light chain and heavy chain Fd' fragments from *E. coli* (19), which is available upon request to the authors. Briefly, the Fab' expression unit is dicistronic with both chains under the transcriptional control of the *pho*A promoter (20), ends with the bacteriophage λ t₀ transcriptional terminator (21) and is cloned between the EcoRI and HindIII site of pBR322 (22). The humanized V$_L$ and V$_H$ domains (12) are precisely fused on their 5' ends to a gene segment encoding the heat-stable enterotoxin II signal sequence (23) and on their 3' side to human κ₁ (C$_L$) (24) and IgG1 (C$_H$1) (25), followed by the hinge sequence CysAlaAla (19) constant domains, respectively. Chimeric and humanized versions of mumAb anti-CD3 Fab' were constructed by precisely replacing gene segments encoding humAb4D5-8 V$_L$ and V$_H$ in pAK19 with appropriate genes encoding mAb anti-CD3 V$_L$ and V$_H$ variants by subcloning (26) and site-directed mutagenesis as described (12). Fab' fragments were expressed in a phage-resistant derivative of *E. coli* strain RV308 (27) at high cell density in the fermentor as previously described (19).

Construction of Bispecific F(ab')₂ Fragments. Intact and functionally active humAb4D5-8 Fab' has been recovered from *E. coli* fermentation pastes with the unpaired hinge cysteine present mainly (75–90%) in the labile-free thiol form (Fab'-SH) by affinity purification using Streptococcal protein G at pH ~5 in the presence of 10 mM EDTA (19). The anti-CD3 mAb Fab'-SH was recovered by similar procedures and reacted with 5,5'-dithiobis (2-

nitrobenzoic acid) (DTNB) (28) to form the thionitrobenzoate derivative (Fab'-TNB). The construction of bispecific (Bs) F(ab')₂ fragments was completed by directed chemical coupling (29) of Fab'-TNB derivative of the anti-CD3 mAb with humAb4D5-8 Fab'-SH. Equimolar quantities of Fab'-TNB (by TNB content) and Fab'-SH (by SH content) were coupled at a combined concentration of ≥0.25 mg/ml in the presence of 100 mM Tris-HCl, pH 7.5, and 10 mM EDTA for 1 h at 37°C. The resulting BsF(ab')₂ fragments were isolated from the coupling reaction by S100-HR gel filtration (Pharmacia Fine Chemicals, Piscataway, NJ) in the presence of PBS. The BsF(ab')₂ samples were passed through a sterile 0.2-μm filter and stored either at 4°C or flash frozen in liquid nitrogen and stored at −70°C until used.

Cell Lines. Breast tumor cell lines SK-BR-3 and MDA-MB-175 were purchased from the American Type Culture Collection (Rockville, MD). NR6/10 cells (NIH 3T3 fibroblasts overexpressing p185^{HER2}) were obtained from Dr. D. Slamon, University of California (Los Angeles, CA). With the exception of MDA-MB-175, these cell lines overexpress HER2/p185^{HER2} as reported (3). The cells were grown in an equal mixture of DME and F12 Ham (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated (30 min. 56°C) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml Streptomycin (Gibco Laboratories) (complete medium).

Human Cytotoxic Lymphocytes. Blood of normal volunteers was drawn into heparinized syringes, mixed with an equal volume of PBS layered onto Ficoll/Hypaque gradient (specific gravity 1.077) and centrifuged for 45 min at 400 g. The separated band of PBMC was aspirated, washed three times in ice cold PBS, and resuspended in complete medium. PBMC were depleted of monocytes by adherence to plastic for 60 min in 100 × 60-mm plates (Costar Corp., Cambridge, MA) at 37°C–5% CO₂. Nonadherent PBMC were activated by incubation in the presence of IL-2 for 24 h and were used as effector CTL against ⁵¹Cr-labeled tumor targets in a 4-h ⁵¹Cr release cytotoxicity assay (30). In some experiments, effector CTLs were tested against targets prepared by PHA blastogenic stimulation of PBMC obtained from the same donor of effector cells as detailed previously (30).

Cytotoxicity Assay. SK-BR-3, or NR6/10 cells (3 × 10⁶/ml), were labeled with 150 μCi of Na₂⁵¹CrO₄ (Amersham Corp., Arlington Heights, IL) for 60 min, washed, adjusted to 10⁴ cells/50 μl of complete medium, and dispensed into round-bottomed microtiter plates containing quadruplicates of various numbers of effector CTLs in 100 μl of complete medium. Various concentrations of Bs F(ab')₂ fragments alone or mixed with p185^{HER2} ECD were then added in 50-μl volumes (final volume per well = 200 μl) and the plates were incubated at 37°C–5% CO₂. After 4 h, the supernatants were harvested (Skatron Inc., Sterling, VA), and their radioactivity was determined using a gamma counter (Micromedic Systems, Inc., Horsham, PA). Percent cytotoxicity was calculated as follows: percent cytotoxicity = 100 × (A − B)/(C − B), where A represents the mean counts per minute (cpm) in test supernatants, B represents the mean cpm in supernatants of targets alone (spontaneous ⁵¹Cr release), and C represents the mean cpm in supernatants of targets lysed with 1% SDS (maximum ⁵¹Cr release).

Flow Cytometric Analysis of BsF(ab')₂ Binding. Aliquots of 10⁶ cells were mixed in either PBS + 1% FCS (PBS + 1%) or PBS + 1% containing chimeric or humanized BsF(ab')₂ (10 μg/ml). The cells were incubated on ice for 45 min, washed twice in PBS + 1%, and stained with fluorescein-labeled goat anti-human Fab (Tago Inc., Burlingame, CA) for 45 min. In experiments involving the blocking of BsF(ab')₂ binding, cells were treated with BsF(ab')₂

antibody in the absence or presence of soluble p185^{HER2} extracellular domain preparation of the p185^{HER2}, or rCD4 receptor as a negative control (50 μg/ml) before addition of the fluorescein-labeled reagent. The stained cells were washed four times in ice-cold PBS + 1% and analyzed using a FACS® (Becton Dickinson & Co., Mountain View, CA). 10⁵ cells were acquired by list mode, and measurements were performed on a single-cell basis and were displayed as frequency distribution histograms. Dead cells and debris were gated out of the analysis on the basis of forward light scatter.

Results

Humanization of mumAb Anti-CD3 V$_L$ and V$_H$. The gene segments encoding mumAb anti-CD3 V$_L$ and V$_H$ were first cloned by PCR from the corresponding hybridoma, UCHT1, and sequenced (Fig. 1). Next, the deduced variable domain amino acid sequences and molecular modeling were used to design a humanized variant of mumAb anti-CD3 (v1) (Fig. 2) as previously described for mumAb4D5 (12). Corresponding genes for humanized anti-CD3 v1 were created by gene conversion mutagenesis (12) starting from humAb4D5 genes and using long preassembled oligonucleotides (Fig. 1). As detailed in Materials and Methods, further humanized anti-CD3 variants were created by replacement of two additional residues from mumAb anti-CD3 with their human counterparts to investigate their role in antigen binding. Thus, humanized anti-CD3 v2 and v3 incorporate the replacements V$_H$ K73D and V$_L$ R53S, respectively, whereas v4 includes both of these changes.

Preparation of BsF(ab')₂ Fragments. We have previously described the secretion of functional humAb4D5 Fab' fragments from E. coli at titers of 1–2 g/liter as judged by antigen-binding ELISA after affinity purification on Staphylococcal protein A (19). Chimeric and humanized versions of anti-CD3 were expressed in the same vector (pAK19) at titers of up to 400 mg/liter as judged by total Ig ELISA. Fab' fragments were recovered from E. coli cell pastes with the hinge cysteine present mainly (75–90%) as the free thiol (Fab'-SH). This was achieved by affinity purification of Fab'-SH on Streptococcal protein G at pH 5 to maintain the thiol in the less reactive protonated form and in the presence of EDTA to chelate metal ions capable of catalyzing disulfide bond formation. Bs F(ab')₂ fragments were then constructed by directed chemical coupling in vitro of humAb4D5 Fab' and anti-CD3 mAb Fab' using the procedure of Brennan et al. (29). One arm of the BsF(ab')₂ was always the most potent humanized anti-p185^{HER2} variant previously identified (humAb4D5-8), which binds p185^{HER2} ECD threefold more tightly than the murine parent Ab (12). The other arm was either a chimeric or humanized variant of the anti-CD3 mAb. Henceforth in this text the term chimeric BsF(ab')₂ refers to a molecule in which one arm is the humanized anti-p185^{HER2} and the other arm is the chimeric anti-CD3; and the terms BsF(ab')₂ v1, v2, v3, and v4 describe a molecule in which one arm is humanized anti-p185^{HER2} and the other arm is humanized anti-CD3 variant 1, 2, 3, and 4, respectively (Fig. 2).

Specific Binding of BsF(ab')₂ Fragments to Cells. Binding of BsF(ab')₂ fragments to NR6/10 cells overexpressing HER2/

A

muMAb anti-CD3 V$_L$

[sequence block A - partially legible nucleotide and amino acid sequences]

Figure 1. Amino acid and nucleotide sequences of mumAb anti-CD3 and humAb anti-CD3 variant 1 V$_L$ (A) and V$_H$ (B). Amino acids are numbered using the scheme of Kabat et al. (16) to accommodate differences in V domain lengths. For example, the residues between V$_H$ positions 82 and 83 are designated 82a, 82b, and 82c, respectively. The 5' end of mumAb anti-CD3 V$_L$ and V$_H$ nucleotide sequences are derived from the corresponding PCR primers and are identified by lower case (see Materials and Methods). Amino acid sequence differences between mumAb and humAb anti-CD3 are shown by asterisks, and residues that were replaced in generating additional humanized variants are indicated by pound signs (see text). The CDR residues according to sequence (16) and structural (39) definitions are shown by overlining and underlining of the CDR label, respectively. Genes encoding humAb anti-CD3 V$_L$ and V$_H$ variant 1 were created by gene conversion mutagenesis (12) of corresponding mumAb4D5 genes using sets of four contiguous oligonucleotides whose 5' and 3' ends are shown by arrows below the sequences (see Materials and Methods).

p185^{HER2} was investigated by flow cytometric analysis (Fig. 3). Chimeric BsF(ab')$_2$ significantly bound to NR6/10 cells as shown by a significant increase in the fluorescence intensity compared with background level (A and B). The presence of p185^{HER2} ECD (50 µg/ml) in the binding assay reduced the binding of chimeric BsF(ab')$_2$ almost to background level (C), whereas addition of an irrelevant soluble receptor (rCD4) at a similar concentration did not interfere with the binding (D). These results demonstrate the specificity of chimeric BsF(ab')$_2$ binding to cells overexpressing p185^{HER2}. Similar results were obtained using BsF(ab')$_2$ v1–v4.

Additional FACS® experiments were performed to quantify the binding of BsF(ab')$_2$ fragments to human CTLs and to breast tumor SK-BR-3 cells. All Bs F(ab')$_2$ fragments bound to SK-BR-3 cells with equal efficiency as anticipated since the anti-p185^{HER2} arm is identical in these molecules

(Fig. 4, left). In contrast, there were significant differences in the binding efficiency of these molecules to human CTLs. Chimeric BsF(ab')$_2$ antibody was most effective in binding (Fig. 4, right, B) followed by BsF(ab')$_2$ v1 (C), v3 (E), and v2 (D), as reflected by different peaks of fluorescence intensity; BsF(ab')$_2$ v4 antibody was least effective in its binding to cytotoxic lymphocytes (F). Thus, the manipulation of the anti-CD3 arm of the BsF(ab')$_2$ fragments profoundly altered its binding to lymphocytes.

Targeting of Tumor Cell Killing by BsF(ab')$_2$ Fragments. The killing of cells overexpressing p185^{HER2} (NR6/10) or breast tumor cells SK-BR-3 by activated human cytotoxic lymphocytes was examined in the presence of various doses of BsF(ab')$_2$, and preliminary results indicated that as little as 10 ng/ml f chimeric BsF(ab')$_2$ or BsF(ab')$_2$ v1 was sufficient to cause maximal enhancement in the cytotoxic activity of CTLs. This dose (10 ng/ml) was used to compare the ability
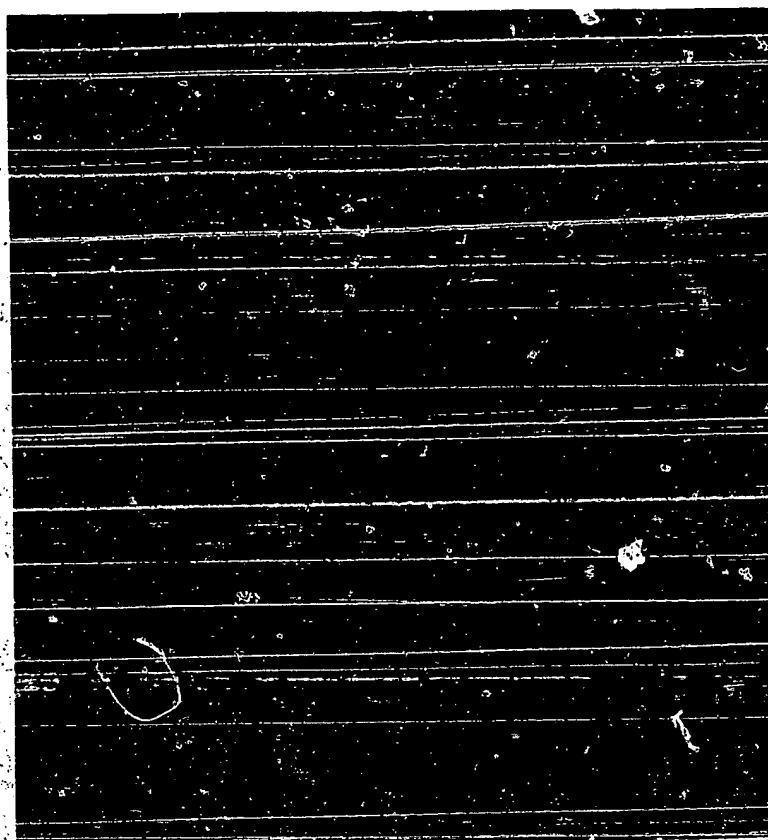
Figure 2. Stereoview of humanized anti-CD3 Variant 1 V_L and V_H domains. An α-carbon trace is shown with side chains of residues that differ between the murine and humanized versions. The six CDRs are labeled, and the side chains of V_L R53 and V_H K73 are represented by space-filling balls.

of chimeric BsF(ab')₂ to enhance the cytotoxic activity of CTL with each of the BsF(ab')₂ variant molecules. The addition of 10 ng/ml of chimeric BsF(ab')₂, BsF(ab')₂ v1, or v3 caused a three- to fourfold enhancement of the cytotoxicity of CTLs against SK-BR-3 breast tumor cells (Fig. 5 A) whereas the presence of BsF(ab')₂ v2 or v4 resulted in no enhancement above control values. Enhancement caused by

Figure 3. Flow cytometric analysis of NR6/10 cells bound to chimeric BsF(ab')₂. Cells were incubated with PBS (A), chimeric BsF(ab')₂ (B), chimeric BsF(ab')₂ mixed with p185^{HER2} ECD (C), or chimeric BsF(ab')₂ mixed with recombinant CD4 (D) before staining with goat anti-human F(ab')₂–FITC (conjugated Ab).

BsF(ab')₂ v1 was reversed by the addition of p185^{HER2} ECD to the assay mixture (Fig. 5 A) demonstrating the specificity of antibody action. The results from an independent experiment (Fig. 5 B) demonstrate that 10 ng/ml of BsF(ab')₂ v1 consistently enhanced the function of CTLs against SK-BR-3 targets known to overexpress p185^{HER2} (64 pg/μg cell protein; 3, 12) but had no effect on the cytotoxicity of CTLs against MDA-MB-175 targets, which express low to moderate levels of p185^{HER2} (7.7 pg/μg cell protein; 3, 12). These results demonstrate the efficacy of chimeric BsF(ab')₂ v1, and v3 in directing cytotoxic lymphocytes to kill breast tumor targets overexpressing p185^{HER2} but not targets with low p185^{HER2} expression. The cytotoxicity data (Fig. 5 A) correlate well with FACS® binding data (Fig. 4) in that BsF(ab')₂ v2 and v4, which were inefficient in binding to cytotoxic lymphocytes, failed to direct tumor cell killing in the cytotoxicity assay. None of these BsF(ab')₂ molecules affected the cytotoxic activity of human CTL when tested against PHA-induced blastogenic targets derived from the same donor, demonstrating that these BsF(ab')₂ do not mediate the lysis of normal autologous lymphoid cells.

Discussion

Considerable progress has been made toward the development of BsmAbs as therapeutic agents for human cancer (reviewed in reference 9). Human CTLs directed with BsmAb
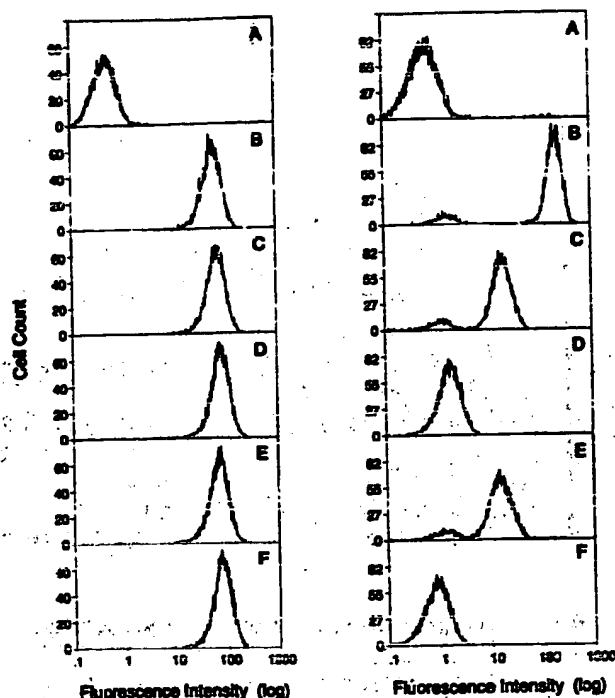
Figure 4. Flow cytometric analysis of the binding of BsF(ab')₂ antibodies to human CTLs and human breast tumor SK-BR-3 cells. Histograms on the *left* illustrate SK-BR-3 cells incubated with PBS (*A*), chimeric BsF(ab')₂ (*B*), BsF(ab')₂ v1 (*C*), v2 (*D*), v3 (*E*), or v4 (*F*) before staining with the FITC-conjugated Ab. Histograms on the *right* illustrate human CTLs incubated with PBS (*A*), chimeric BsF(ab')₂ (*B*), BsF(ab')₂ v1 (*C*), v2 (*D*), v3 (*E*), or v4 (*F*) before staining with FITC conjugated Ab.

have been shown to block the growth of human tumor xenografts in nude mice (31, 32). In other studies involving carcinoma patients, local lysis of tumor cells was observed after infusion of T cells activated with BsmAb (8). In addition, the efficacy of antitumor associated antigen × anti-CD3-bispecific antibody in the management of malignant glioma

has been reported (33). A major drawback for the application of murine mAbs has been the elicitation of an immune response after repeated administration into humans. The humanization f BsmAb may reduce the immunogenicity of these reagents, thus avoiding possible untoward effects in human subjects (10, 11, 34).

The development of biologically active fully humanized BsF(ab')₂ fragments as demonstrated in this study has not been reported previously. We have used an efficient *E. coli* expression system (19) for the production of humanized Fab' molecules with anti-p185^HER2 and anti-CD3 specificities. The Fab' molecules were recovered with the unpaired hinge cysteine present as the free thiol and used to form the BsF(ab')₂ by directed chemical coupling in vitro (29). The approaches used in this study obviate the inherent problems in generating Fab'-SH from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield, as well as partial reduction that is not completely selective for the hinge disulfide bonds (29). Furthermore, by engineering the hinge region to leave a single cysteine residue, we prevent intrahinge disulfide bonding without resorting to the use of highly toxic arsenite to chelate vicinal thiols (29). The purified BsF(ab')₂ antibody fragments are reactive with human T cells and cells overexpressing p185^HER2. The ability of these BsF(ab')₂ to mediate targeted killing of tumor cells correlated well with their efficiency of binding to CTLs as revealed by FACS® analysis.

The humanization of the anti-CD3 arm (as in v1) resulted in a decrease in the binding efficiency to CTL but did not alter the ability of the molecule to enhance CTL cytotoxicity against tumor targets at the lowest BsF(ab')₂ concentration studied (10 ng/ml). The observation that <2% occupancy by antibody is sufficient to trigger T cell activation (35) together with the high degree of purity of the BsF(ab')₂ used may explain the observed potency of the humanized version in mediating tumor cell killing at pharmacological concentrations (10 ng/ml).
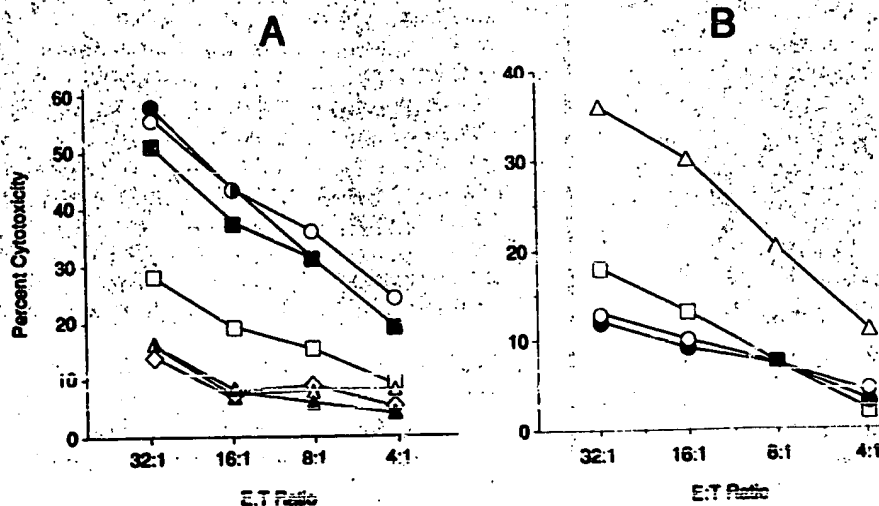
Replacement of the murine residue V₁ 53 with serine

Figure 5. Targeting of breast tumor cell killing by BsF(ab')₂. ⁵¹Cr-labeled SK-BR-3 targets (*T*) were co-incubated with effector (*E*) CTLs at different E/T ratios for 4 h. In *A*, percent cytotoxicity was calculated based on ⁵¹Cr release in cultures with no antibody added (▲), in the presence of 10 ng/ml of chimeric BsF(ab')₂ (●), BsF(ab')₂ v1 (○), v2 (◇), v3 (■), v4 (△), or v1 + p185^HER2ECD (□). In *B*, two different ⁵¹Cr-labeled target cells were used. MDA-MB-175 targets tested in the absence (○) or presence (●) of 10 ng/ml of BsF(ab')₂ v1, and SK-BR-3 targets tested in the absence (□) or presence (△) of 10 ng/ml of BsF(ab')₂ v1. The MDA-MB-175 cells express low to moderate level of p185^HER2 as quantitatively state in Results.

(human residue) in humanized anti-CD3 v1 to create v3 resulted in little or no change in the binding efficiency to CTL, suggesting that V_L R53 is probably not an essential antigen-binding determinant. The binding efficiency of v1, however, was severely reduced upon the replacement of V_H K73 with the human counterpart, Asp, in FR3 to make v2. Further reduction of the binding to CTLs was observed when V_L R53 in v2 was replaced by Ser to create v4, whose binding capacity to CTLs was almost completely abolished. Given the fact that V_H K73 is outside of the CDRs, these results imply that contact between selected amino acid residues in FRs with other residues in adjacent CDRs or direct interaction with antigen can influence the antigen-binding efficiency and specificity of the hypervariable loops (11, 36). However, additional amino acid replacements are required to determine whether murine residue V_H K73 is an important binding determinant or whether the human residue D73 compromises binding. Nevertheless, these data demonstrate that amino acid residues outside CDRs should be considered in mAb humanization. Additional amino acid replacements are currently being installed in the humanized anti-CD3 Fab' in an effort to improve its binding efficiency. A three-dimensional molecular model of the humanized (v1) anti-CD3 arm V_L and V_H domains is presented (Fig. 2), illustrating the side chains of residues that differ between murine and humanized versions of anti-CD3 arm. This figure shows that V_H K73 in FR3 is located in a loop proximal to CDRs H1 and H2.

The fact that a number of adenocarcinomas are characterized by an overexpression of p185^{HER2} presents a unique opportunity for testing the feasibility as well as the efficacy of targeted tumor immunotherapy whereby patients CTLs can be redirected with BsmAb for tumor killing. Fully humanized BsF(ab')$_2$ fragments are shown here to be effective mediators of human breast tumor target lysis in vitro at pharmacologically relevant concentrations. We are currently investigating whether these molecules may be capable of targeting breast tumor cells in vivo for destruction by CTLs. The fact that these molecules are fully humanized and, therefore, less likely to elicit an immune response in cancer patients further advances their potential use in targeted immunotherapy. Importantly, the BsF(ab')$_2$ molecules failed to mediate the killing of normal lymphoid cells (PHA-blasts) or even tumor cells expressing only low to moderate levels of p185^{HER2}.

The mechanism(s) by which CTLs cause the lysis of tumor targets are not known. However, it has been reported that the cytolytic activity is induced by the binding of antibody-target conjugates to specific receptors on the effector cell surface (6). One view proposes that the crosslinking by BsmAbs between clusters of receptors on tumor targets and triggering structures on immune effector cells can induce the release of effector cytolytic substances including cytolysin which may contribute to target killing (8). Crosslinking may also activate T cells for production of cytokines, e.g., TNF-α and IFN-γ, both of which can exert cytotoxic effects on tumor cells in vitro. The data in this report are consistent with the crucial requirement for crosslinking in order to trigger effector killing of tumor target. Thus two of the BsF(ab')$_2$ variants, v2 and v4, shown to be perfectly capable of binding to target cells but not to effector CTLs, failed to direct tumor killing in the cytotoxicity assay. Further, the presence of p185^{HER2} ECD, which is shown to block BsF(ab')$_2$ binding to the target, caused a marked inhibition of directed CTL killing of tumor targets.

Our E. coli Fab' expression system in combination with directed chemical coupling as described here has proven effective in the production of clinically relevant quantities of functional humanized antibody fragments. The availability of purified material should facilitate the initiation of clinical studies to evaluate the efficacy of BsF(ab')$_2$ in redirecting CTL killing of tumor cells. It should be noted that the use of F(ab')$_2$ fragments should permit for a more efficient tissue penetration in vivo (37). The systems used here also allow for replacements of amino acid residues in CDRs and FRs making it possible to study structure-function relationships among the different variant Ab fragments.

Collectively, the data presented here demonstrate the feasibility of producing genetically engineered fully humanized BsF(ab')$_2$ shown to be biologically active in two different in vitro assays. The expression systems described can be applied efficiently for the production of Fab' molecules with selected specificities, and offer an opportunity for understanding the structure-function relationship among the produced Ab fragments. These and similar studies will advance the potential use of BsmAbs in targeted immunotherapy of cancer in humans.

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Please send me the following references ASAP:

Journal of Experimental Medicine 175:217-225 (Jan. 1, 1992)

Int. J. Cancer 50:800-804 (1992)

Int. J. Cancer, Suppl. (1992), 7(Bispecific Antibodies Targeted Cell Cytotoxic.), 42-4

PROC AM ASSOC CANCER RES ANNU MEET. (1992) 33 (0), 344

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Cancer Immunol. Immunother. (1994), 39(1), 41-8

Proceedings of the American Association for Cancer Research Annual Meeting, (1994) Vol. 35, No. 0, pp. 219

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Publication of the International Union Against Cancer
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HUMAN c-erbB-2 PROTO-ONCOGENE PRODUCT AS A TARGET FOR BISPECIFIC-ANTIBODY-DIRECTED ADOPTIVE TUMOR IMMUNOTHERAPY

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To develop an efficient strategy for the targeting of anti-tumor effector cells, we prepared bispecific antibody (BsAb) containing anti-CD3 and an anti-c-erbB-2 proto-oncogene product. The prepared BsAb specifically reacts with both c-erbB-2-positive tumor cells and CD3+ CTL. Human CD4+ helper/killer T cells, induced from peripheral-blood mononuclear cells by activation with immobilized anti-CD3 monoclonal antibody (MAb) plus IL-2, showed no significant cytotoxicity against tumor cells. However, treatment of human CD4+ helper/killer cells with the BsAb caused the induction of specific cytotoxicity against c-erbB-2-positive tumor cells. CD4+ helper/killer cells also produced significant amounts of IL-2 during co-culture with c-erbB-2-positive tumor cells in the presence of the BsAb. Moreover, by combination with the BsAb, CD4+ helper/killer cells showed a strong in vivo anti-tumor effect against c-erbB-2 transfectant or human colon-cancer cells implanted in nude mice. Our results strongly suggest that the c-erbB-2 proto-oncogene product on human tumor cells may be a good target for BsAb-directed adoptive tumor immunotherapy.

It has been reported that adoptive immunotherapy using IL-2-activated killer cells is a useful strategy for tumor therapy in both animal and human systems (Mule et al., 1984; Nishimura et al., 1986; Rosenberg et al., 1985). One of the major problems of adoptive tumor immunotherapy has been the difficulty of targeting effector cells to the tumor sites. However, recent development of bispecific antibody (BsAb), which reacts with both killer and target cells, has made it possible to deliver anti-tumor effector cells specifically to target cells (Perez et al., 1985; Staerz et al., 1985; Franger et al., 1991). Several experimental and clinical studies have shown that adoptive tumor immunotherapy combined with anti-CD3 × anti-tumor BsAb was an effective strategy for tumor therapy (Nitta et al., 1990a; Titus et al., 1987). This BsAb-directed adoptive tumor immunotherapy may become a common protocol for clinical application, if good target molecules, widely distributed on tumor cells, can be found.

Our colleagues have produced MAbs recognizing the extracellular domain of the human c-erbB-2 proto-oncogene products (Masuko et al., 1989). The c-erbB-2 gene and its product are amplified in a variety of adenocarcinomas, including breast, gastric and colon cancer (Slamon et al., 1987; Semba et al., 1985; Hudziak et al., 1989; Yokota et al., 1986). Therefore, we supposed that BsAb containing anti-c-erbB-2 and anti-CD3 might become a good tool for the therapy of human cancer.

In this study we investigated the targeting of CD4+ helper/killer T cells induced by immobilized anti-CD3 plus IL-2 using anti-CD3 × anti-c-erbB-2 BsAb. The BsAb can trigger both helper and killer functions of CD4+ helper/killer cells in vitro. Moreover, BsAb-directed CD4+ helper/killer cells showed a strong inhibitory effect on the growth of c-erbB-2-positive human tumor cells implanted in nude mice. These results strongly suggested that the human c-erbB-2 proto-oncogene product may become a good target for BsAb-directed adoptive tumor immunotherapy in humans.

MATERIAL AND METHODS

Monoclonal antibody

Hybridoma cells producing OKT3 or OKT4 MAb were purchased from ATCC (Rockville, MD). SER-4 MAb recognizing the p185-kDa extracellular domain of the c-erbB-2 gene product was produced by Dr. T. Masuko from the mice immunized with SK-BR-3 breast cancer cells (Masuko et al., 1989).

Cells

NIH-3T3, SV11 (NIH-3T3 cells transfected with normal c-erbB-2 gene), A4-15 (NIH-3T3 cells transfected with mutated c-erbB-2 gene), RAScD (NIH-3T3 cells transfected with EJ ras gene), KATOIII (human gastric cancer), LS174T (human colon cancer; kindly donated by Dr. H. Fukamachi, Tokyo University, Tokyo) SK-BR-3 (human mammary cancer) Molt-4F (human T-lymphoma cells) and Daudi (human B-lymphoma cells) were used for the experiments. The characteristics of oncogene transfectants were described in previous paper (Masuko et al., 1989). All the cells were maintained in RPMI1640 medium supplemented with glutamine, penicillin, streptomycin, HEPES buffer and 10% heat-inactivated FCS.

Preparation of BsAb containing anti-CD3 and anti-c-erbB-2

The BsAb containing anti-CD3 and anti-c-erbB-2 were prepared from OKT3 and SER-4 MAbs as reported (Nitta et al., 1989, 1990b). Briefly, F(ab')₂ fragments prepared from the 2 MAbs by treatment with pepsin or papain were reduced to Fab-SH by incubation with 0.5 mM dithiothreitol (DTT) in 0.1 M PBS for 30 min at room temperature. OKT3-Fab-S-NB derivatives were further prepared from Fab-SH derivatives by incubation with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in PBS for 30 min at room temperature. OKT3-Fab-S-NB was mixed with SER-4-Fab-SH derivatives at a 1:1 ratio and incubated for 4 hr at 37°C. Then, anti-CD3 × anti-c-erbB-2 BsAb was purified by FPLC chromatography (Pharmacia, Uppsala, Sweden).

Generation of CD4+ helper/killer cells

CD4+ helper/killer cells were induced from peripheral blood mononuclear cells (PBMC) of breast tumor patients. Briefly, CD4+ T cells were freshly isolated from PBMC of tumor patients by using FACStar (Becton Dickinson, Mountain View, CA) and then the cells were cultured in the presence of recombinant IL-2 (2,000 U/ml) and immobilized anti-CD3 MAb for 10-14 days. Anti-CD3 MAb was immobilized to plastic plates by incubation of the plates with 5 μg/ml of anti-CD3 MAb for 1 hr at 37°C. Over 98% of the activated cells were CD3+CD4+ T cells and used as CD4+ helper/killer cells. The activated CD4+ helper/killer cells could be expanded for long periods (over 50 days) by periodic restimula-

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tion with IL-2 plus immobilized anti-CD3 MAb (Nishimura *et al.*, 1991).

Flow cytometry

Analysis of cell-surface markers was carried out by FACS-can using a Consort 30 program. Details of staining and sorting have been reported (Nishimura *et al.*, 1990). Fluorescence data were collected with logarithmic amplification. For each sample, data from 10,000 volume-gated viable cells were collected.

IL-2 assay

CD4⁺ helper/killer cells (10^6/ml) were cultured with A4-15 c-*erbB*-2 transfectant (10^5/ml) in the presence or absence of

Fluorescence intensity

FIGURE 1 – Reactivity of anti-c-*erbB*-2 SER-4 MAb with various cell lines. The reactivity of SER-4 MAb was determined by flow cytometry as described in the text. *(a)* NIH-3T3 cells; *(b)* A4-15 c-*erbB*-2 transfectant; *(c)* SV11 c-*erbB*-2 transfectant; *(d)* RAScD EJras transfectant; *(e)* LS174T colon cancer; *(f)* KATOIII gastric cancer; *(g)* SK-BR-3 breast cancer; *(h)* Molt-4F T lymphoma; *(i)* Daudi B lymphoma.

Fluorescence intensity

FIGURE 2 – Preparation and reactivity of anti-CD3 × anti-c-*erbB*-2 bispecific antibody (BsAb). BsAb was construted from F(ab')₂ fragments of 2 MAbs (OKT3 and SER-4) as described in the text. The reaction scheme is illustrated in the right panel. The reactivity of the prepared BsAb was analyzed by FACScan using CD4⁺ helper/killer cells *(a)* or c-*erbB*-2 transfectant, A4-15 *(b)*. a, control unstained profile; b, profile of the cells stained with native OKT3 MAb; c, profile of the cells stained with native SER-4 MAb; d, profile of the cells stained with BsAb.
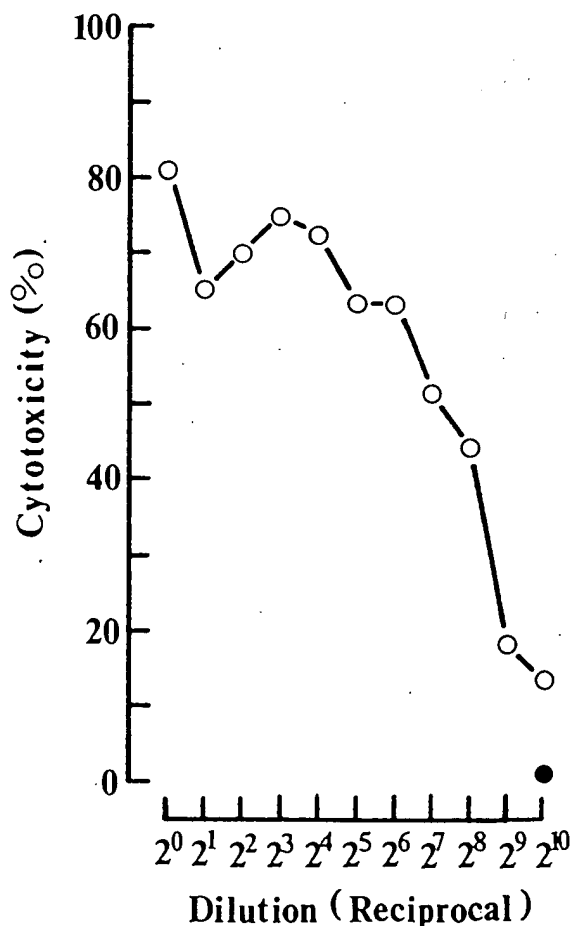
BsAb (0.5 µg/ml) for 24 hr. After incubation, the culture supernatants were harvested from the plate and their IL-2 activity was determined using the IL-2-dependent HT-2 cell line. Briefly, HT-2 cells (10^4/well) were cultured with diluted samples for 24 hr and then pulsed with ^{3}H-TdR (0.5 µCi/well) for 4 hr. The growth of HT-2 cells in culture supernatants from CD4⁺ helper/killer cells was strongly blocked by anti-IL-2 MAb but not by anti-IL-4 MAb (data not shown).

Cytotoxicity assay

The cytotoxicity of CD4⁺ helper/killer cells was determined by the 4-hr ^{51}Cr-release assay in the presence or absence of anti-CD3 × anti-c-*erbB*-2 BsAb (0.5 µg/ml). Percentage cytotoxicity was calculated as reported by Nishimura *et al.* (1987).

In vivo *neutralization assay*

The *in vivo* anti-tumor activity of CD4⁺ helper/killer cells was determined by Winn's (1961) assay using c-*erbB*-2 transfectant, EJras transfectant or LS174T human colon cancer cells. Briefly, tumor cells (2×10^6) were injected i.d. into the abdomen of BALB/c nude mice with or without BsAb (10 µg), CD4⁺ helper/killer cells (2×10^7) or CD4⁺ helper/killer cells plus BsAb. The growth of tumor was determined by measuring tumor size in perpendicular diameters. Ten nude mice were used for each group.

FIGURE 3 – The anti-CD3 × anti-c-*erbB*-2 BsAb induced CD4⁺ helper/killer-cell-mediated cytotoxicity in a dose-dependent manner. The cytotoxicity of CD4⁺ helper/killer cells against A4-15 c-*erbB*-2 transfectant was determined by a 4-hr ^{51}Cr-release assay in the presence (O) or absence ( ) of various concentrations of diluted SER-4 MAb. SER-4 MAb was diluted 2-fold from 2 µg/ml. Effector to target ratio was 20:1.

RESULTS

The SER-4 MAb recognizing the 185-kDa c-erbB-2 gene product specifically reacted with c-erbB-2 gene transfectants (SV11 and A4-15) but not with untransfected NIH-3T3 cells or EJras transfectant (RAScD). Moreover, SER-4 MAb showed strong reactivity with some human tumor cell lines such as KATOIII, SK-BR-3 and LS174T but not with non-epithelial tumor such as Daudi B lymphoma and Molt-4F T lymphoma cells (Fig. 1). According to the method previously reported (Nitta et al., 1989, 1990b), both anti-CD3 Fab'-S-NB derivative and anti-erbB-2 Fab'-SH derivative were prepared and then coupled to construct F(ab')₂ BSAb containing anti-CD3 and anti-c-erbB-2. As shown in Figure 2, the prepared BsAb reacted with both c-erbB-2 transfected and CD3⁺CD4⁺ helper/killer cells. However, the BsAb showed no reactivity with either c-erbB-2-negative tumor cells or CD3⁻ killer cells (data not shown).

Using the prepared BsAb, we next tried in vitro targeting of CD4⁺ helper/killer cells to c-erbB-2-expressing transfectants or LS174T human colon cancer cells. CD4⁺ helper/killer cells were induced from freshly isolated CD4⁺ T cells of tumor patients by activation with immobilized anti-CD3 MAb plus recombinant IL-2. The activated CD4⁺ helper/killer cells showed marginal cytotoxicity against various target cells. However, addition of BSAb into the culture resulted in the induction of a strong cytotoxicity against c-erbB-2 transfectants (A4-15) in a dose-dependent manner (Fig. 3). The BsAb alone had no cytotoxic effect on the target cells (data not shown). To determine the specificity of BsAb-treated CD4⁺ helper/killer cells, we next investigated the cytotoxicity of CD4⁺ helper/killer cells against c-erbB-2-positive or -negative cell lines. As shown in Figure 4, CD4⁺ helper/killer cells lysed c-erbB-2 transfectants (SV11 and A4-15) and c-erbB-2-expressing tumor

cells (KATOIII and LS174T) by targeting with BsAb. However, native NIH-3T3, EJras-transfectant (RAScD) or c-erbB-2-negative Daudi B-lymphoma cells were resistant to CD4⁺ helper/killer cells even in the presence of BsAb. Thus, F(ab')₂ BsAb containing anti-CD3 plus anti-c-erbB-2 enabled us to carry out specific targeting of CD4⁺ helper/killer cells in vitro. Consistent with the lack of CD4⁺ helper/killer-cell-mediated cytotoxicity in the absence of BsAb, IL-2 production of CD4⁺ helper killer cells was not induced by co-culture with c-erbB-2-expressing A4-15 cells. However, if CD4⁺ helper/killer cells were bridged with A4-15 cells by the addition of BsAb, significant IL-2 production was observed in the culture supernatants (Fig. 5).

Finally, we investigated the in vivo anti-tumor effect of CD4⁺ helper/killer cells in combination with BsAb. The results are illustrated in Figure 6. When c-erbB-2 transfectant (A4-15) was inoculated i.d. into the abdomen of nude mice, the cells grew rapidly. In contrast, injection of A4-15 cells with CD4⁺ helper/killer cells and BsAb resulted in a marked inhibition of cell growth. No such in vivo anti-tumor effect was observed when A4-15 cells were inoculated with BsAb alone or CD4⁺ helper/killer cells alone, and no BsAb-directed in vivo anti-tumor effects of CD4⁺ helper/killer cells were demonstrated in RAScD transfectant, indicating that anti-CD3 × anti-c-erbB-2 BsAb specifically delivered CD4⁺ helper/killer cells to c-erbB-2-expressing tumor cells in vivo. Interestingly, the growth of c-erbB-2-expressing human colon cancer (LS174T) was also completely blocked by BsAb-directed CD4⁺ helper/killer therapy.

FIGURE 4 – Specific in vitro targeting of CD4⁺ helper/killer cells using BsAb. CD4⁺ T cells isolated from peripheral blood mononuclear cells of breast cancer patients using FACStar were cultured with immobilized anti-CD3 plus recombinant IL-2 for 14 days. The cytotoxicity of activated CD4⁺ helper/killer cells was determined by the 4-hr ⁵¹Cr-release assay in the presence (hatched columns) or absence (open columns) of anti-CD3 × anti-c-erbB-2 BsAb (0.5 μg/ml). Effector to target ratio was 20:1. Oncogene transfectants (SV11, A4-15, RAScD) and human tumor cell lines (KATOIII, Daudi B lymphoma, LS174T) were used as target cells. The cell-surface expression of c-erbB-2 was determined by FACScan.
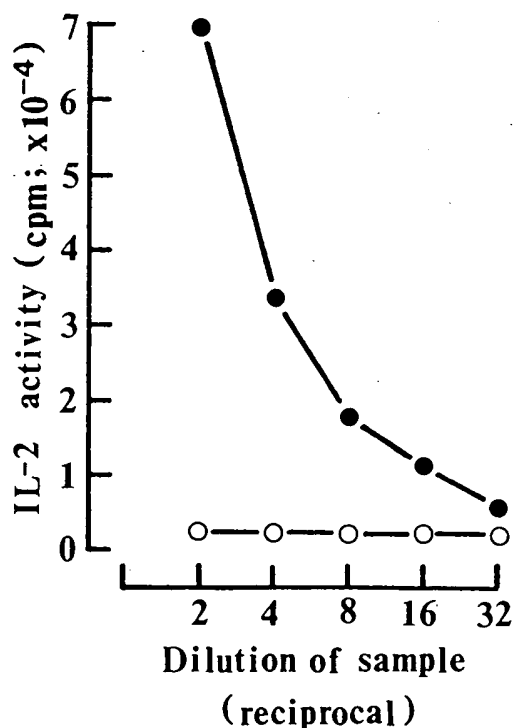

FIGURE 5 – BsAb can trigger the IL-2 production of CD4⁺ helper/killer cells. CD4⁺ helper/killer cells were co-cultured with A4-15 cells in the presence ( ) or absence (O) of BsAb (0.5 μg/ml) for 20 hr at 37°C. After incubation, the IL-2 activity of the culture supernatants was measured using HT-2 cell lines as described in the text. The ³H-TdR incorporation (cpm) of HT-2 cells is shown. Mean ± SE of triplicate samples is indicated.
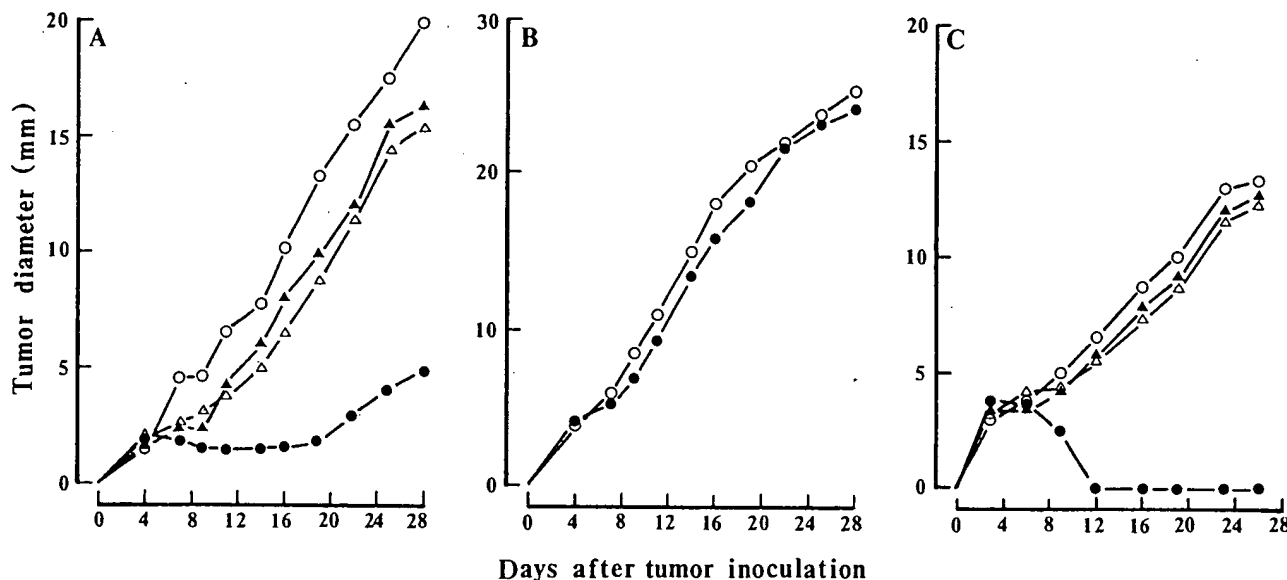
FIGURE 6 – BsAb-directed *in vivo* anti-tumor effect of CD4⁺ helper/killer cells. The *in vivo* anti-tumor effect of CD4⁺ helper/killer cells was examined by Winn's assay using c-erbB-2 transfectant, A4-15 *(a)*, EJras transfectant, RAScD *(b)* or LS174T human colon cancer cells *(c)*. Tumor cells (2 × 10⁶) were inoculated i.d. into the abdomen of nude mice with or without (O) CD4⁺ helper/killer cells (2 × 10⁷) plus BsAb (10 μg) (●), CD4⁺ helper/killer cells alone (△) or BsAb alone (▲). The tumor diameter shown is the mean of 2 perpendicular diameters. Ten nude mice per group were used for the experiment.

DISCUSSION

In this report, we present an efficient strategy for human cancer therapy using anti-CD3 × anti-c-erbB-2 BsAb and CD4⁺ helper/killer cells. Since the CD3 molecule is a key molecule for the triggering of T-cell functions (van Seventer *et al.,* 1987, Hoffman *et al.,* 1985), anti-CD3 × anti-tumor BsAb can activate the cytotoxic machinery of CTL in addition to bridging the effector cells to the tumor target cells (Perez *et al.,* 1985; Staerz *et al.,* 1985; Franger *et al.,* 1991). Nitta *et al.* (1990*a*) reported that targeting therapy using LAK cells treated with anti-CD3 × anti-glioma BsAb was more successful than currently used LAK therapy (Rosenberg *et al.,* 1985). Several other investigators have also demonstrated the efficacy of BsAb-directed adoptive tumor immunotherapy in animal models (Titus *et al.,* 1987). These results strongly indicate that targeting adoptive immunotherapy might become a widely available tool for human cancer therapy if we can produce an MAb which reacts with cell-surface molecules expressed on a variety of human tumor cells.

This report indicates that MAb recognizing a human c-erbB-2 proto-oncogene product is a good candidate for use in BsAb-directed adoptive tumor immunotherapy. It has been shown that the c-erbB-2 gene and gene product are amplified in a variety of human adenocarcinoma cells and the amplification of the gene is correlated with poor clinical prognosis for certain cancer patients (Slamon *et al.,* 1987; Semba *et al.,* 1985; Hudziak *et al.,* 1989; Yokota *et al.,* 1986). As shown in Figure 1, SER-4 MAb recognizing the p185 c-erbB-2 gene product reacts with c-erbB-2 transfectants such as SV11 and A4-15 but not native NIH-3T3 or other transfectants. Moreover, gastric, colon and breast-cancer cell lines were also reactive with SER-4 MAb.

In the present study, we prepared F(ab')₂ BsAb containing anti-CD3 and anti-c-erbB-2 (Fig. 2) and applied this BsAb to the targeting of CD4⁺ T cells which have both helper and killer functions. CD4⁺ T cells possess both helper and killer activity at the clonal level (Fleisher and Wagner, 1986; Moretta, 1983).

Moreover, several groups have reported that activation of T cells with immobilized anti-CD3 MAb caused the induction of CD4⁺ CTL as well as CD8⁺ CTL (Jung *et al.,* 1987; Garrido *et al.,* 1990). Although freshly isolated CD4⁺ T cells cannot directly respond to IL-2 because of the lack of p75 IL-2 receptor (Nakamura *et al.,* 1991), we found that activation of freshly isolated CD4⁺ T cells with immobilized anti-CD3 plus IL-2 resulted in the proliferation and generation of CD4⁺ helper/killer T cells which have both IL-2-producing activity and cytotoxic activity (Nishimura *et al.,* 1991, 1992). If the CD4⁺ helper/killer cells were targeted to local tumor sites by combination with BsAb, it would become possible to augment "local help" to facilitate the activation of anti-tumor effector cells at local tumor sites. As illustrated in Figures 3 and 4, the addition of BsAb containing anti-CD3 plus anti-c-erbB-2 resulted in the specific targeting of CD4⁺ helper/killer cells to c-erbB-2-expressing transfectants and human tumor cells *in vitro*. Interestingly, CD4⁺ helper/killer cells produced significant amounts of IL-2 during co-culture with c-erbB-2-expressing tumor cells in the presence of the BsAb (Fig. 5). These results suggest that BsAb-treated CD4⁺ helper/killer cells may act effectively as both killer and IL-2 producing cells at targeted tumor sites.

Using oncogene transfectants and LS174T human colon cancer which were implanted into nude mice, we also investigated the therapeutic efficacy of BsAb-treated CD4⁺ killer cells. As clearly indicated in Figure 6, CD4⁺ helper/killer cells showed a strong anti-tumor activity against c-erbB-2 transfectant or c-erbB-2-expressing LS174T human colon cancer by combination with anti-CD3 × anti-cerbB-2 BsAb. However, no therapeutic effect of BsAb-directed CD4⁺ helper/killer cells was demonstrated against EJras transfectant, indicating that the BsAb specifically targeted CD4⁺ helper/killer cells *in vivo*.

Thus, our data show that the human c-erbB-2 proto-oncogene product might become a good target molecule for BsAb-directed adoptive tumor immunotherapy and confirm

that CD4$^+$ helper/killer cells will provide an efficient strategy for BsAb-directed adoptive tumor immunotherapy in humans. We have already established a large-scale culture system of CD4$^+$ helper/killer cells. Therefore, it will be possible to submit this BsAb-directed CD4$^+$ helper/killer therapy to clinical trial after extensive checking of any side-effects of anti-CD3 × anti-c-erbB-2 BsAb.

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Journal of Experimental Medicine 175:217-225 (Jan. 1, 1992)

Int. J. Cancer 50:800-804 (1992)

Int. J. Cancer, Suppl. (1992), 7(Bispecific Antibodies Targeted Cell Cytotoxic.), 42-4

PROC AM ASSOC CANCER RES ANNU MEET. (1992) 33 (0), 344

INTERNATIONAL JOURNAL OF BIOLOGICAL MARKERS, (1993 Oct-Dec) 8 (4) 233-9

Cancer Immunol. Immunother. (1994), 39(1), 41-8

Proceedings of the American Association for Cancer Research Annual Meeting, (1994) Vol. 35, No. 0, pp. 219

Thanks,

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Article title	Possible targets on carcinoma for bMAb retargeting of lymphocyte or drug cytotoxicity
Article identifier	002071369200484L
Authors	Canevari_S Mezzanzanica_D Menard_S Ferrini_S Moretta_L Colnaghi_M_I
Journal title	International Journal of Cancer
ISSN	0020-7136
Publisher	Wiley-Liss
Year of publication	1992
Volume	-
Issue	SUPPL.
Supplement	1
Page range	42-44
Number of pages	3
User name	Adonis
Cost centre	Development
PCC	$17.50
Date and time	Monday, February 25, 2002 2:34:04 PM

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

POSSIBLE TARGETS ON CARCINOMA FOR bMAb RETARGETING OF LYMPHOCYTE OR DRUG CYTOTOXICITY

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Retargeting of drugs or lymphocyte cytotoxicity through bi-specific monoclonal antibodies (bMAbs) has been proven a therapeutic tool against human carcinoma both in pre-clinical *in vitro* and *in vivo* studies. Some of these reagents have already been introduced into clinical trials and preliminary results appear to be promising. However, improvement of the specificity of this approach could be achieved by selecting more suitable target molecules on tumor cells. The research focused on developing MAbs directed against molecules with tumor-restricted distribution and homogeneous expression. Cell-membrane receptors for nutrients or growth factors which operationally represent tumor-specific molecules due to their overexpression, could be considered appropriate targets. Several bMAb anti-nutrients (the folate-binding protein) or growth-factor receptor (c-erbB1, c-erbB2)/anti-triggering molecules have been generated and all were able to efficiently retarget the relevant population of lymphocytes on tumor cells. In order to design a more reliable and selective therapeutic tool, the following parameters were analyzed: correlation between cyto-toxicity and antigen level, potency and possible modulation of the target molecule.
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CHOICE OF SUITABLE TARGET STRUCTURE ON HUMAN CARCINOMA

After the initial demonstration, about 10 years ago, that bifunctional monoclonal antibodies (bMAbs) could specifically direct the cytotoxic activity of the ricin toxin against tumor cells (Raso, 1982), bMAbs were exploited to efficiently re-target cytotoxic lymphocytes of any specificity against selected target cells (Perez *et al.*, 1985). In an attempt to develop therapeutic tools against human carcinomas, which might be more selective than conventional chemo- or radio-therapeutic agents, the bMAb methodology has been widely applied. In the majority of the initial reports, the selected target molecules were those recognized by the first generation of anti-tumor MAbs, *i.e.*, onco-fetal, differentiation or generically defined cancer-associated antigens (Table I). The bMAbs thus obtained have been found to be very potent both in pre-clinical *in vitro* and in *in vivo* studies, and some of these reagents have already been introduced into clinical trials. Although the results appear to be promising, the selected targets present 2 major limitations: they are expressed at a relatively high level on some normal tissues, whereas within a single tumor they are not present on all cells.

Improvement of the specificity of bMAb re-targeting could be achieved by selecting more suitable target molecules on tumor cells with tumor-restricted distribution and homogeneous expression. Some particular cell-surface molecules, such as receptors for hormones, nutrients and growth factors, have been suggested as being appropriate targets for antibody-driven therapy (Harris, 1990). Although they are expressed at a very low level on some normal tissues, their over-expression on tumor cells endows them with an   perational tumor specificity. Among nutrient receptors, the folate-binding protein (FBP) is homogeneously over-expressed in almost all ovary carcinomas (Coney *et al.*, 1991). Several bMAb anti-FBP/anti-triggering molecules have been generated either by the hybrid-hybridoma technique or by chemical reassociation. All of them were able to specifically target the relevant population

of lymphocytes (Tαβ, Tγδ, NK) against ovary carcinoma (Lanzavecchia and Scheidegger, 1987; Mezzanzanica *et al.*, 1988; Ferrini *et al.*, 1989, 1991). For the anti-FBP/anti-CD3 biMAb, after a complete validation in pre-clinical short-term and long-term *in vivo* studies (Mezzanzanica *et al.*, 1991) and toxicity evaluation in a phase-I clinical trial (Mezzanzanica, 1991), a phase-II clinical trial is now in progress.

For growth-factor receptors, the c-erbB1 and c-erbB2 proteins were found to be homogeneously over-expressed in about one third of breast and ovary tumors, the former being expressed in a majority of glioma and squamous-cell carcin - mas. Such over-expression has been correlated with rapid relapse and poor survival (Rilke *et al.*, 1991).

An anti-c-erbB1 MAb has been used in our laboratory t generate bMAbs either with anti-CD3 (data not shown) or anti-doxorubicin MAbs (Balsari *et al.*, 1988) using conventional technology. Another research group has reported the generation of bMAb anti-c-erbB2/anti-CD3 using molecular engineering (Shalaby *et al.*, 1992). All these reagents appeared active in *in vitro* pre-clinical evaluation.

EVALUATION OF SOME CRITICAL ISSUES

With the aim of designing a more reliable and selective therapeutic tool, 2 bMAbs were selected. Both reagents, obtained in our laboratory using the hybrid hybridoma technique, were used in purified form after removal of the parental species. The anti-FBP/anti-CD3 was kindly provided by Dr. S. Warnaar (Centocor, Leiden, The Netherlands) as bi-F(ab')2, whereas the anti c-erbB1/anti-CD3 was used as an intact bMAb. These bMAbs were used to analyze several aspects relevant to the specificity of determination of anti-tumor cytotoxicity, *i.e.*, antigenic expression, potency and possible modulation of the target structure.

As reported in Table II, both bMAbs at nanomolar concentration in the presence of CTL clones promoted specific lysis only of those cell lines which expressed the relevant Ag. However, when the level of cytotoxicity was compared to the number of antigenic sites/cell, no direct correlation was observed. On the contrary, when the same anti-c-erbB1 MAb was used alone or after conjugation to alpha sarcin, good correlation between the inhibition of cell proliferation and the antigenic level was observed (Tosi *et al.*, 1992). Moreover, a bMAb anti-c-erbB1/anti-doxorubicin, although less active than the bMAb anti-c-erbB1/anti-CD3, exhibited a certain degree of correlation (data not shown). The different mechanisms of action involved in these therapeutic approaches probably account for the observed differences.

The potency of bMAbs in CTL re-targeting was compared with that of other MAb-driven therapeutic approaches. Although the experimental systems adopted to evaluate their activity were quite different, the relative potencies were compared on a MAb molar basis. As shown in Table III, the anti-FBP MAb according to the very low affinity of murine IgG1 for human Fc receptors was unable to mediate ADCC.

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TABLE I – TARGET STRUCTURES ON HUMAN CARCINOMA CELLS FOR bMAb RE-TARGETING

Category	Distribution on relevant cancers	Partner MAb for re-targeting	Level of analysis	References
Oncofetal differentiation Ag				
CEA	Heterogeneous, on colon, breast, lung carcinoma	Anti-Vinca alkaloid	Pre-clinical *in vivo*	Smith *et al.*, 1990
N-CAM	Heterogeneous, on gliomas, small-cell lung carcinomas	Anti-CD3	Clinical trial	Nitta *et al.*, 1990
Cancer-associated Ag				
Ca-G250	Homogeneous, on renal carcinoma	Anti-CD3	Preclinical *in vivo*	Van Dijk *et al.*, 1991
gp40	Heterogeneous, on various carcinomas	Anti-CD3	Pre-clinical *in vivo*	Barr *et al.*, 1989
			Clinical trial	De Leij *et al.*, 1991
gp72	Heterogeneous, on various carcinomas and sarcomas	Anti-MTX	Pre-clinical *in vitro*	Pimm *et al.*, 1990
3e6/CAMA1	Heterogeneous, on breast carcinomas	Anti-CD3	Pre-clinical *in vivo*	Nelson *et al.*, 1991
Ca19-9	Heterogeneous, on colon and pancreatic carcinomas	Anti-CD16	Pre-clinical *in vitro*	De Palazzo *et al.*, 1990
Nutrient receptors				
FBP	Homogeneous, on ovarian carcinomas	Anti-TCR Anti-CD16	Pre-clinical *in vitro*	Ferrini *et al.*, 1989 Ferrini *et al.*, 1991
		Anti-CD3	Clinical trial	Mezzanzanica *et al.*, 1991
Growth factor receptors				
c-erbB1	Homogeneous, on head and neck, ovarian, breast carcinomas, gliomas	Anti-CD3 Anti-doxorubicin	Pre-clinical *in vitro*	
c-erbB2	Homogeneous, on breast, ovarian and bladder carcinomas	Anti-CD3	Pre-clinical *in vitro*	Shalaby *et al.*, 1992

TABLE II – SPECIFICITY OF CTL RE-TARGETING AND CORRELATION WITH THE ANTIGENIC LEVEL IN THE CASE OF bMAbs DIRECTED AGAINST FBP OF c-erbB1

Parameter	Variable	Percentage lysis in the presence of CTL and bMAb[1]	
		Anti-FBP/ anti-CD3	Anti-c-erbB1/ anti-CD3
Specificity	Target cells[2]		
	Relevant Ag+	60	50
	Relevant Ag–	6	1
Correlation with amount of antigen	Number of sites/cell[3]		
	>1 × 10^6	55	50
	2 × 10^5	80	ND
	7 × 10^4	82	45
	3 × 10^4	ND	75
	Undetectable	12	2

[1]bi F(ab')$_2$ or intact bMAbs at 100 ng/ml were used to retarget CTL clones against the reported target cells at an effector/target ratio of 10:1 in a standard ^{51}Cr-release assay.–[2]The expression of the relevant Ag was monitored by MAb binding. In the case of FBP the relevant Ag+ line was IGROV1 (ovarian carcinoma) and the relevant Ag– line was Mewo (melanoma). In the case of c-erbB1, the relevant Ag+ line was NIH 3T3 HEGFR (mouse fibroblasts transfected with hEGFR cDNA kindly provided by Dr. DiFiore, NIH Bethesda, MD) and the relevant Ag– line was NIH 3T3 (mouse fibroblasts).–[3]The number of sites/cell was determined by analyzing the binding data of the relevant ^{125}I ligand (MOv18 MAb for FBP, EGF for c-erbB1), using Scatchard analysis. The lines used for FBP were: NIH OVCAR3 (4.5 × 10^6 sites/cell); OVCA432 (2 × 10^5 sites/cell); SW626 (7 × 10^4 sites/cell) and U937 (number of sites/cell undetectable). The lines used for c-erbB1 were: A431 (1.4 × 10^6 sites/cell); IGROV1 (3 × 10^4 sites/cell); U87 (6.7 × 10^4 sites/cell); Raji (number of sites/cell undetectable).

On the contrary, the chimeric version of the MAb (kindly provided by Dr. L. Coney, Centocor Malvern, PA), in which the murine constant regions were replaced by human γ1 and k regions, efficiently mediated ADCC in the presence of PBMC. However, on a molar basis, bMAb re-targeting of T-cell

TABLE III – POTENCY OF TUMOR-CELL ELIMINATION: COMPARISON OF DIFFERENT MAb-DRIVEN THERAPEUTIC APPROACHES

Target structure	Therapeutic approach	MAb concentration needed to eliminate 50% of relevant tumor cells
FBP[1]	bMAb + CTL	5.3 × 10^{-12} M
	ADCC murine MAb	Not applicable[3]
	ADCC chimeric MAb	1.3 × 10^{-10} M
c-erbB1[2]	bMAb + CTL	6.6 × 10^{-10} M
	immunotoxin	5.0 × 10^{-10} M

[1]Lysis of the IGROV1 cell line in the presence of a CTL clone (E/T 10:1) and the bMAb anti-FBP/anti-CD3 or of fresh PBMC (E/T 50:1) and the murine MAb against the FBP (mIgG$_1$κ) or its Vmurine/C human chimeric version (hIgG$_1$κ) in a standard ^{51}Cr-release assay.–[2]Lysis of the A431 cell line in the presence of a CTL clone (E/T 10:1) and bMAb anti-c-erbB1/anti-CD3 in a standard ^{51}Cr-release assay and inhibition of A431 protein synthesis in the presence of the immunotoxin anti-c-erbB1 MAb-α sarcin in a standard aminoacid incorporation assay.–[3]Less than 10% lysis, even at the maximum concentration tested (10^{-8}M).

cytotoxicity was about 20 times more efficient than the chimeric MAb-mediated ADCC. In the case of the c-erbB1 target structure, bMAb re-targeting was compared with the activity of the immunotoxin. As reported in Table III, the 2 therapeutic agents exhibited similar potency.

Finally, the possible influence of the target-structure modulation on bMAb cytotoxicity was analyzed. The 2 target structures studied had different internalization behavior upon parental MAb binding. The binding of the anti-FBP MAb to its antigen induces slow and unreliable internalization, whereas that of the anti-c-erbB1 MAb promotes prompt and reliable modulation of about one third of the antigenic sites (Mezzanzanica *et al.*, 1988; Tosi *et al.*, 1992). In order to evaluate the relevance of the MAb bivalence, the internalization ability of 2 monovalent forms of the parental anti-c-erbB1 MAb was tested. The F(ab') fragment was unable to induce internalization (data not shown), whereas the bMAb anti-c-erbB1/anti-doxorubicin almost completely maintained its ability to induce antigen modulation (data not shown). Except for their internal-

ization behavi ur, the 2 target structures were similarly sensitive to bMAb re-targeted lysis (Table II). Similar efficiency may be ascribed to the known absence of correlation between cytotoxicity and the number of antigenic sites/cell.

Cell-membrane receptors for nutrients (FBP) and growth factors (*c-erb*B1 and *c-erb*B2 oncoproteins) may be considered as appropriate targets for bMAb re-targeting of T-cell cytotoxicity due to their homogeneous overexpression on a relevant percentage of epithelial human tumors. Their use for loco-regional treatment of relevant tumors may thus be considered feasible.

Our findings illustrate how much further efforts are needed to develop better predictive *in vitro* tests and suitable *in vivo* models in order to mimic as closely as possible the distribution of the target structure in human patients.

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Journal of Experimental Medicine 175:217-225 (Jan. 1, 1992)

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6 042173

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PROC AM ASSOC CANCER RES ANNU MEET. (1992) 33 (0), 344

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Proceedings of the American Association for Cancer Research Annual Meeting, (1994) Vol. 35, No. 0, pp. 219

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Immunohistochemical detection of c-erbB-2 expression by neoplastic human tissue using monospecific and bispecific monoclonal antibodies

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ABSTRACT: *Selected murine monoclonal antibodies (MAb) have been shown to inhibit relevant tumor growth in vitro and in animal models. Recently, bispecific antibodies (BsMAb) have been developed which target cytolytic effector cells via one antibody binding site and tumor antigen by the other specificity. For example, the BsMAb 2B1 possesses specificity for c-erbB-2 and FcγRIII, the low affinity Fcγ receptor expressed by polymorphonuclear leukocytes (PMN), macrophages and large granular lymphocytes (LGL). The human homologue of the rat neu oncogene, c-erbB-2, has been demonstrated to be amplified in breast, gastrointestinal, lung and ovarian carcinomas. Tumor expression of c-erbB-2 has been shown to be an important prognostic indicator in breast and ovarian carcinomas. The restricted expression of the c-erbB-2 protooncogene product in normal human tissues and the wide distribution of c-erbB-2 expression in such tumors may justify attempts to use an appropriately constructed BsMAb in clinical trials. In this report we have addressed this issue by immunohistochemically evaluating the expression of c-erbB-2 oncogene product in a variety of malignant tumors utilizing 2B1 and the anti-c-erbB-2 monovalent parent of 2B1, 520C9. Among the studied neoplasms, c-erbB-2 expression was detected in 49% of primary carcinomas stained with 520C9 and in 39% of those stained with 2B1. In the group of metastatic tumors, c-erbB-2 oncoprotein was detected in 52% of cases by 520C9 and in 41% by 2B1. Our results indicate that immunocytochemistry using bispecific monoclonal 2B1 is a reliable method for the detection of c-erbB-2 expression, and that this BsMAb detects c-erbB-2 expression in tumors nearly as well as its anti-c-erbB-2 monovalent parent antibody. (Int J Biol Markers, 1993; 8: 233-9)*

KEY WORDS: *C-erbB-2 expression, Oncogenes, Monoclonal antibodies*

INTRODUCTION

Protooncogenes are cellular genes with fundamental roles in cell growth, development and differentiation. They can be activated (oncogenes) by several molecular mechanisms. Numerous studies have shown that oncogenes are involved in human cancer both in the initial conversion of normal to malignant cells and in tumor progression (1). The protooncogene designated c-erbB-2 (HER2/neu) is a cellular oncogene whose product is a transmembrane type MW 185,000 glycoprotein (p185) with tyrosine kinase activity; p185 is also a growth factor receptor for an as yet unidentified ligand (2-5).

Slamon et al have shown that the prognostic value of c-erbB-2 gene amplification in breast cancer may be independent of other well-recognized prognostic factors such as hormonal receptor status and lymph node-positive disease (6). Recently, it has been suggested that, as in breast cancer, overexpression of c-erbB-2 in ovarian cancer might be associated with poor survival (13). Overexpression of c-erbB-2 has also been reported in other human adenocarcinomas of the salivary gland, colon, stomach and lung (2, 7-12, 14), but its roles in the progression or prognosis of these neoplasms remains speculative.

Analyses by immunocytochemistry using MAbs that recognize distinct epitopes of the extracellular domain of p185 have been reported in a variety of normal and transformed human tissues (15-17), but to our knowledge only monospecific antibodies have been utilized. Hybrid antibodies containing two differ-

ent antigen-binding sites may be produced by chemical recombination or from the fusion of two hybridoma lines. The latter method was employed for the production of the bispecific monoclonal antibody (BsMAb) 2B1, which is the fusion product of hybridomas 520C9 (murine IgG_1 recognizing an extracellular domain epitope of c-erbB-2) and 3G8 (murine IgG_1 recognizing an extracellular domain epitope of human FcγRIII) (16).

The goal of the present study was to determine if the bispecific antibody retained the ability of its parental anti c-erbB-2 antibody, 520C9, to recognize the expression of this oncogene product by malignant neoplasms. A secondary objective was to examine the spectrum of malignancies in which 2B1 therapy might be employed to determine the likelihood of identifying suitable candidates in each malignancy. We have examined 126 malignant tumors by immunocytochemical analysis of frozen sections and analyzed the relationship between detection of c-erbB-2 by 520C9 and 2B1. Our findings suggest that 2B1 is equivalent to 520C9 in its ability to recognize c-erbB-2, and that c-erbB-2 expression may increase in metastatic adenocarcinomas of the colon, breast and lung.

MATERIAL AND METHODS

Tissue: The tissues used in this study included ovarian, gastric, lung, colon and metastatic carcinomas. They were obtained from the tumor bank of the Fox Chase Cancer Center, Department of Pathology. A total of 126 tumors were used for the study.

Monoclonal Antibodies: Monoclonal antibody 520C9 (murine IgG_1 recognizing the protooncogene product c-erbB-2) and bispecific monoclonal antibody 2B1 were generously provided by Dr. David Ring, (Chiron Corporation, Emeryville, CA). Briefly, hybrid hybridoma 2B1 was developed by somatic cell fusion of 520C9 and 3G8 (murine IgG_1 recognizing human FcγRIII), as described (16). The active bispecific antibody was purified from the hybridoma supernatant containing the bispecific antibody and its parental antibodies by sequential ion exchange chromatography, yielding greater than 99% pure bispecific antibody (16).

Immunocytochemistry: Localization of the c-erbB-2 proto-oncogene product was evaluated using serial frozen sections. Sections were obtained using a Hacker Bught OTS-AS-EC-MR Cryostat. Histological evaluation of each tumor specimen was performed on a section stained with hematoxylin and eosin. Monoclonal antibody (AE1/AE3) to cytokeratin

(Biogenex Laboratories, San Jose, CA) was used to confirm the presence of viable tumor cells in the section.

A negative control slide incubated with non-specific mouse IgG (Sigma Immunochemicals, St. Louis, MO) was examined to assess non-specific binding. Duplicate slides for each case were stained with 520C9 (10μg/ml) and 2B1 (10μg/ml). As a positive control, 2B1 and 520C9 were used to stain cells of the SK-OV-3 line (American Type Culture Collection, Rockville, MD); these cells express high levels of c-erbB-2 message and protein.

The Stravigen Super Sensitive Alkaline Phosphatase Immunostaining Kit (Biogenex Laboratories) was used for all the immunohistology procedures. Five μm frozen sections were mounted onto poly-L-lysine-coated slides, fixed for 15 minutes in cold acetone and dried at room temperature for 2 hours. Sections were blocked for 30 minutes with normal mouse ascites to diminish non-specific binding. Slides then were incubated overnight at 4°C with monoclonal antibodies 2B1, 520C9 and controls.

Then the slides were washed with PBS 2% BSA, incubated for 30 min at room temperature with goat anti-mouse antibody (Stravigen Super Sensitive Alkaline Phosphatase Kit), and washed. Substrate was added (Fast Red) and the slides were incubated 5 minutes, washed and counterstained with Meyer's hematoxylin solution (Sigma, St. Louis, MO). The slides were independently evaluated by two pathologists. The intensity of staining was graded as: 0, no greater than the negative control; 1+, weak; 2+, moderate; and 3+ equal to or greater than positive control.

RESULTS

A total of 101 primary lung, breast, colon and ovarian neoplasms and 25 metastases from diverse neoplasms were stained using bispecific antibody 2B1 and parental antibody 520C9. C-erbB-2 expression was detected in 41% of the tumors stained with 2B1 while 520C9 detected 52% of all cases. Table I shows the percentage of cases stained with 520C9 and 2B1 in each tumor group, and Table II shows the distribution of staining intensity for each studied tumor type. Overexpression of c-erbB-2 was defined as the presence of an immunostaining signal at the surface membranes of the malignant epithelium. The signal was represented by a distinct chromogen staining. In all cases only membrane staining was considered.

Table III shows the 2B1 and 520C9 staining pat- .

TABLE I - DETECTION OF c-erbB-2 BY IMMUNOHISTOCHEMI-
CAL ANALYSIS USING 520C9 AND 2B1

Summary of Immunohistochemical Reactivity
for 2B1 and 520C9

Tissue source (Total# samples)	Positive Samples 2B1 # samples (%)	520C9 # samples (%)
Lung (25)	7 (28%)	9 (36%)
Breast (25)	9 (36%)	13 (52%)
Colon (25)	12 (48%)	14 (56%)
Ovary (26)	12 (46%)	14 (53%)
Metastases (25)	12 (48%)	15 (60%)
Total	52 (41%)	65 (52%)

terns, related to the histological classification of the tumors. Of the 25 cases of breast carcinoma stained with 2B1 and 520C9, 20 were classified as invasive ductal carcinoma (IDC). Of these, 8 cases showed binding by 2B1 and 11 cases by 520C9. All cases that stained with either antibody showed a comedo pattern characterized by centrally necrotic ducts with higher nuclear and mitotic grade. In all these cases, the tumor was mostly composed of an invasive component. The three cases of IDC that failed to stain with 2B1, but were positive with 520C9, were classified as high grade. Of the IDC cases that did not stain with 520C9 (9) or 2B1 (12), all were characterized by a histological pattern of moderate tubule formation or a predominant papillary and cribiform pattern with low to moderate nuclear and mitotic grade.

When the *in situ* component of IDC was analyzed,

TABLE II - RESULTS OF IMMUNOHISTOCHEMICAL STAINING INTENSITY OF PRIMARY NEOPLASMS USING BISPECIFIC 2B1 AND PARENTAL MONOCLONAL 520C9

Tissue source (total # of samples)	Immunohistochemical Grading Intensity 520C9	2B1	# per group
Ovary (26)	+++	+++	(5)
	+++	++	(2)
	++	++	(3)
	++	-	(2)
	+	-	(2)
	-	+	(2)
	-	-	(10)
Total	14	12	
Lung (25)	+++	+++	(4)
	++	++	(2)
	++	-	(1)
	+	+	(1)
	+	-	(1)
	-	-	(16)
Total	9	7	
Breast (25)	+++	+++	(2)
	++	++	(5)
	+	+	(2)
	+	-	(4)
	-	-	(12)
Total	13	9	
Colon (25)	+++	+++	(5)
	++	++	(4)
	++	+	(1)
	++	-	(2)
	+	+	(2)
	-	-	(11)
Total	14	12	

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TABLE III - NUMBER OF CASES STAINED WITH 2B1 AND 520C9 CLASSIFIED BY HISTOLOGICAL TYPE

# of Cases		# of Reactive Tumors	
		2B1	520C9
	Ovary:		
15	Papillary serous carcinomas	7	7
4	Papillary mucinous adenocarcinomas	2	3
4	Endometrioid adenocarcinomas	2	3
3	Undifferentiated	1	1
Total 26		12	14
	Lung:		
17	Adenocarcinomas	5	5
5	Squamous cell carcinomas	2	4
2	Small cell carcinoma	0	0
1	Bronchioalveolar carcinoma	0	0
Total 25		7	9
	Breast		
20	Ductal (invasive)	8	11
3	Ductal (in situ)	1	2
1	Lobular (invasive)	0	0
1	Lobular (in situ)	0	0
Total 25		9	13
	Colon		
5	Well differentiated adenocarcinomas	2	3
13	Moderately differentiated adenocarcinomas	8	10
6	Undifferentiated adenocarcinomas	2	1
1	Adenocarcinoid	0	0
Total 25		12	14

all cases that stained with 2B1 or 520C9 showed binding to the *in situ* ductal carcinoma (ISDC). In three cases, ISDC was the only component of the tumor; two stained positively with 520C9, and one was stained by 2B1. The only two examined lobular carcinomas (one classified as *in situ* and one invasive) failed to stain with either antibody.

The pattern of staining in breast cancer specimens was rather homogenous. In most positive cases, between 70 and 80% of the cells stained with both antibodies, even in the groups classified as *in situ*. A few tumor sections contained adjacent normal tissue. Normal breast ducts immediately adjacent to the carcinomas stained with both 520C9 and 2B1, although the intensity of staining was very weak when compared with the tumor.

When the colon carcinomas were studied, of the total (n = 20), 14 were detected by 520C9 and 12 by 2B1. In this group, those classified as moderately differentiated carcinomas were best detected with both antibodies. In the colon carcinoma group, staining was observed not only in the membrane but also in cytoplasm and was mostly homogeneous. A high percentage (50-60%) of cells within the tumor stained with both antibodies. Two cases had normal glands immediately adjacent to the tumor and binding was detected in both cases with 520C9 and 2B1. However, the intensity of staining in either case was very weak compared with the tumor.

Lung carcinomas were characterized by a focal pattern of staining. Only one case of squamous carcinoma showed diffuse staining. In all cases the stroma was not bound by either antibody, binding to normal lung structures was not observed (scattered, minimal staining of bronchial epithelial cells was noted with both antibodies). When 2B1 was used, alveolar macrophages were stained by that antibody. Of a total of 17 lung adenocarcinomas, 5 were stained by 2B1 and 520C9. The pattern of staining was rather similar for both antibodies in this group, which was characterized by a focal pattern comprising between 20 to 30% of the tumor cells. In the squamous carcinoma group 520C9 detected 4 cases out of 5 while 2B1 detected 2 out of 5. In this group, a higher percentage (50-60%) of tumor cells were stained with both antibodies, when compared to adenocarcinomas. Small cell carcinomas (n = 2) were not stained by either antibody.

In ovarian cancer, 14 of 26 specimens were detected by 520C9 while 2B1 detected 12 of 26. In all cases where staining was present, it was confined to the epithelial

TABLE IV - PATTERNS OF 2B1 AND 520C9 REACTIVITY IN METASTATIC TUMOR SAMPLES

Tissue source (# of samples)	Immunohistochemical Grading Intensity		# per group
	520C9	2B1	
Colon (10)	+++	+++	(5)
	++	++	(2)
	+	-	(1)
	-	-	(2)
Breast (8)	+++	++	(3)
	+++	-	(1)
	++	++	(1)
	-	-	(3)
Unknown (5)	+++	++	(1)
	+++	-	(1)
	-	-	(3)
Ovary (1)	++	++	(1)
Appendix (1)	-	-	(1)

component. Papillary serous carcinomas were found to stain heavily with both antibodies while the other histological patterns demonstrated a less intense reaction. In all positive samples, a homogeneous pattern of staining characterized by membrane staining of every cell in the tumor was dominant.

Immunohistochemically detectable c-erbB-2 oncogene was noted in normal ovarian epithelium in 5 cases of 6 examined. In these cases, every epithelial cell was stained but the intensity was very low. In all cases, adjacent ovarian stroma was not bound by the antibodies.

The detection of metastasis by 2B1 and 520C9 showed patterns of staining similar to their corresponding primary tumors. However, a higher degree of intensity and percentage of stained cells was observed in this group when compared with the primary lesions. Table IV shows patterns of reactivity in the tumor samples.

DISCUSSION

For BsMAb to be therapeutically useful, it is necessary that the expression of the tumor antigen to be targeted is largely restricted to neoplasms. The c-erbB-2 oncogene product is an attractive candidate target antigen for several reasons. The expression patterns of this antigen can be molecularly defined and correlated with protein expression by a variety of techniques. Thus, the sensitivity and specificity of an antibody's detection capabilities can be more easily defined. The expression of this antigen by a significant percentage of common neoplasms makes it a potentially generally useful target. As expression of this protein may be associated with poor prognosis, the targeting and elimination of c-erbB-2 positive cells may be particularly useful. Finally, the importance of this protein in regulating tumor growth is suggested by the ability of several anti-c-erbB-2 antibodies to inhibit growth of relevant neolasms *in vitro* and in appropriate animal models.

The studies presented in this report confirm previous examination of c-erbB-2 expression by human neoplasms, and extend previous findings. The increased expression of c-erbB-2 by metastatic lesions observed in these studies may have been due to the small number of the metastases which were examined. It must be pointed out that matched pairs of primary and metastatic lesions were not available for these studies from the same patient. However, if confirmed, these results suggest that therapeutic strategies targeting c-erbB-2 may directly attack a biologically important target in the setting of metastatic disease.

2B1 has a number of characteristics that suggest it may have therapeutic potential. These include potent redirection of cytotoxicity by large granular lymphocytes and macrophages (19), and substantial antitumor effects on human xenografts in immunodeficient mouse models (20). The studies presented in this report confirm that 2B1 recognizes c-erbB-2 expression by human neoplasms nearly as well as 520C9. Thus, the

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minor reductions in c-erbB-2 detection by 2B1, manifested either by less staining intensity or the identification of fewer c-erbB-2 lesions, can be attributed to the reduced avidity of the BsMAb compared with 520C9. The process of producing 2B1 therefore had little effect on the ability of its 520C9-derived portion to bind c-erbB-2. Based on these results, 2B1 is sufficiently capable of detecting c-erbB-2 expression by human neoplasms to justify its continued evaluation as a therapeutic reagent.

ACKNOWLEDGEMENT

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Journal of Experimental Medicine 175:217-225 (Jan. 1, 1992)

Int. J. Cancer 50:800-804 (1992)

Int. J. Cancer, Suppl. (1992), 7(Bispecific Antibodies Targeted Cell Cytotoxic.), 42-4

PROC AM ASSOC CANCER RES ANNU MEET. (1992) 33 (0), 344

INTERNATIONAL JOURNAL OF BIOLOGICAL MARKERS, (1993 Oct-Dec) 8 (4) 233-9

Cancer Immunol. Immunother. (1994), 39(1), 41-8

Proceedings of the American Association for Cancer Research Annual Meeting, (1994) Vol. 35, No. 0, pp. 219

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Article title	Antigen forks: bispecific reagents that inhibit cell growth by binding selected pairs of tumor antigens
Article identifier	034070049410036A
Authors	Ring_D_B Hsieh_Ma_S_T Shi_T Reeder_J
Journal title	Cancer Immunology Immunotherapy
ISSN	0340-7004
Publisher	Springer
Year of publication	1994
Volume	39
Issue	1
Supplement	0
Page range	41–48
Number of pages	8
User name	Adonis
Cost centre	Development
PCC	$25.00
Date and time	Monday, February 25, 2002 12:52:56 PM

Antigen forks: bispecific reagents that inhibit cell growth by binding selected pairs of tumor antigens

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Abstract. Bispecific antibodies of a new category, termed "antigen forks", were constructed by crosslinking antibodies that recognized pairs of distinct tumor cell surface antigens. At concentrations of 1–100 nM, several such forks inhibited the growth of human tumor cell lines bearing both relevant antigens. The same cells were not inhibited by unconjugated component antibodies, and the active conjugates did not inhibit the growth of human cell lines that expressed lower levels of relevant antigens. The three most active antigen forks all contained monoclonal antibody 454A12, which recognizes human transferrin receptor. This antibody was conjugated respectively to antibodies 113F1 (against a tumor-associated glycoprotein complex), 317G5 (against a 42-kDa tumor-associated glycoprotein), or 520C9 (against the c-erbB-2 protooncogene product). The 317G5-454A12 fork strongly inhibited the HT-29 and SW948 human colorectal cancer cell lines, while the 113F1-454A12 and 520C9-454A12 forks strongly inhibited the SK-BR-3 human breast cancer cell line and the 113F1-454A12 fork was also effective against SW948. By designing forks against antigens of incompatible function that are co-expressed at high levels on tumor cells but not on normal tissues, it may be possible to generate reagents that inhibit tumor growth with enhanced selectivity.

Key words: Bispecific antibodies – Tumor-associated antigens – Cell growth inhibition

Introduction

Many monoclonal antibodies recognizing human tumor cell surface antigens are now available. More often than not, binding of such antibodies to tumor cells has little effect on cell function, unless the antibody is linked to a cytotoxic

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moiety such as a drug, toxin or radioactive isotope. In some cases, antibody binding alone may initiate or block signal transduction, leading to a change in cell state, or may alter antigen turnover by enhancing or inhibiting endocytosis or changing the route of intracellular processing. The effect of antibody binding may vary according to valency. Binding of monovalent antibody fragments often has little or no effect, implying that antigen crosslinking significantly affects the consequences of binding.

Some antibodies that bind to cell surface antigens have direct effects on tumor cell growth. For instance, some monoclonal antibodies to the c-erbB-2 protooncogene product inhibit tumor cell proliferation in vitro and tumor xenograft growth in animal models [1, 13, 27–30]. Antibodies to transferrin receptor inhibit cell growth, alone [33, 35, 36] or in combination with the iron-chelating drug deferoxamine [14, 15, 37]. Antibodies to the Apo-1 antigen and the Fas oncogene product trigger apoptosis in a variety of cells, leading to programmed cell death [32, 39], and antibodies to P-glycoprotein have been reported to increase the drug sensitivity of multidrug-resistant tumor cells [8]. Such antiproliferative or cytotoxic effects on tumor cells may result from a number of mechanisms, including blockage of growth signals or down modulation of growth factor receptors, triggering of programmed death or differentiation signals, blockage of nutrient transport, or blockage of toxin excretion.

While examples can be found of antibodies that strongly inhibit tumor growth, and also of antibodies that are highly selective for tumors as opposed to normal tissues, the availability of antibodies that meet both criteria is limited. To circumvent this scarcity, we speculated that bispecific antibodies recognizing two distinct tumor antigens with different properties would be more likely to disrupt cellular function than a single antibody, and could also be more tumor-selective if antigens with little overlap of expression on normal tissues were chosen. Because of their ability to bind two antigens simultaneously, and to distinguish them from bispecific antibodies that direct labels or effector moieties to a target cell, we refer to such reagents as "antigen forks".

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The most obvious way to construct an antigen fork is as a bispecific antibody (although forks could also be built using ligand or receptor subunits, peptides or even small molecules as binding elements). Bispecific antibodies may be chemically synthesized as antibody heteroconjugates by covalently attaching two monoclonal antibodies [12] or monoclonal antibody fragments [4, 7] with different antigenic specificities. Alternatively, bispecific antibodies may be produced from hybrid hybridomas [20, 21, 24]. In order to test whether any "antigen fork" bispecific antibodies will actually inhibit tumor cell growth, we have constructed simple chemically crosslinked whole-antibody conjugates from a set of six monoclonal antibodies recognizing distinct tumor surface antigens.

Materials and methods

Antibodies and cell lines. Murine IgG monoclonal antibodies 2G3, 113F1, 260F9, 317G5, 454A12 and 520C9 were raised by immunizing Balb/c mice with human breast cancer membranes or cell lines [5]. The isotype, immunogen and antigenic specificity of these antibodies is shown in Table 1, along with comments on antigen function (if known). Cell lines were obtained as follows: the HT-29 human colorectal and SK-BR-3 human breast cancer cell lines as gifts from the late Dr. J. Fogh of the Sloan Kettering Institute for Cancer Research; the HBL-100 human mammary cell line from the Mason Research Institute (collection now transferred to the American Type Culture Collection); the SW948 human colorectal cancer cell line from Dr. Louis M. Weiner of the Fox Chase Cancer Center; the SK-OV-3 human ovarian cancer cell line from the American Type Culture Collection.

Flow cytometry. Samples containing 1×10^6 cells were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (PBS/BSA) and incubated for 30 min at 4° C in PBS/BSA containing first antibody at a final dilution of 20 µg/ml. After washing three times in PBS/BSA, the cells were incubated with fluorescein-iso-thiocyanate(FITC)-conjugated F(ab')2 fragment of goat anti-(mouse IgG Fc) (Jackson ImmunoResearch, West Grove, Pa.) for another 30 min at 4° C. The last wash contained 50 µg/ml propidium iodide to stain dead cells. Samples were analyzed on an EPICS V cell sorter (Coulter Electronics. Hialeah, Fla.). Dead cells and cellular debris were eliminated based on their forward-angle light scatter and red fluorescence. At least 20000 live cells were scored for each sample and the intensity of green fluorescence was measured on a logarithmic scale.

SPDP derivatization of antibodies. The two antibodies to be coupled were separately concentrated to 10–20 mg/ml using a Centricon 100 apparatus (Amicon Inc., Beverly, Mass.), and dialysed into coupling buffer (100 mM KPO4, 100 mM NaCl, pH 7.5). Each antibody was stirred for 3 h at room temperature with a sixfold molar excess of SPDP [N-succinimidyl-3-(2-pyridyldithio)propionate; Pierce, Rockford, Ill.] pre-dissolved at 6.25 mg/ml in dimethylsulfoxide before adding to the antibody solution. Excess SPDP was removed by chromatographing the antibody solutions over PD10 columns (Pharmacia Biotech, Piscataway, N.J.). Antibody A was chromatographed in acetate buffer (100 mM sodium acetate, 100 mM NaCl, pH 4.5) and antibody B in PBS (20 mM NaPO4, 150 mM NaCl, 1 mM EDTA, 0.02% NaN3, pH 7.2). In either case, fractions were monitored by absorbance at 280 nm and protein-containing fractions were pooled.

Antibody coupling. Derivatized antibody A in acetate buffer was brought to 40 mM dithiothreitol (Sigma Chemical, St. Louis, Mo.) by addition of 24 mg/ml dithiothreitol in acetate buffer, and stirred 30 min at room temperature. It was then chromatographed on a PD10 column in PBS to remove excess dithiothreitol, and peak fractions were again pooled on the basis of their absorbance at 280 nm. Antibody A was then immediately combined with antibody B (in 1:1 molar ratio), and coupling was allowed to proceed for 4 h at room temperature. At the end of this incubation, the reaction was stopped and excess free thiol groups were blocked by addition of 100 mg/ml iodoacetamide (1 mg iodoacetamide to a reaction containing 10 mg each antibody).

Heteroconjugate purification. The reaction mixture was centrifuged for 30 s at 12000 rpm in an Eppendorf microfuge, applied to a Bio-Sil TSK400 HPLC column (BioRad, Richmond, Calif.) equilibrated in PBS, and eluted at room temperature with PBS at a flow rate of 0.7 ml/min, collecting 0.375-ml fractions and monitoring absorbance at 280 nm. Fractions were analyzed by nonreduced sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) on 4%–15% gradient Phast gels (Pharmacia). Fractions containing monomeric immunoglobulin were discarded, and fractions containing dimers and low oligomers were pooled for further use.

MTT assay for cell growth. Samples containing 5000–10000 target cells in 100 µl growth medium (Isove's medium +10% heat-inactivated fetal bovine serum +2 mM glutamine +50 µg/ml gentamycin) were seeded in triplicate in 96-well flat-bottom tissue-culture plates, and incubated overnight at 37° C in 5% CO2. Serial twofold dilutions of antibodies were made in the wells leaving 100 µl final volume per well, and the plates were incubated for 3–6 days. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (CellTiter 96, no. G4100, Promega Corp., Madison, Wis.) was used to evaluate the number of viable cells remaining in the wells. A 15-µl sample of dye solution was added per well and the plate was incubated

Table 1. Antigen fork component antibodies

Antibody	Immunogen	Isotype	Antigen	Function
2G3	Breast cancer membranes	IgG1	High-molecular-mass mucin	Modulation of adhesion?
113F1	MCF-7 breast cancer cell line	IgG3	40/60/100/200 kDa glyco-protein complex	Unknown
260F9	Breast cancer membranes from liver metastasis	IgG1	55 kDa glycoprotein	Unknown
317G5	BT-20 breast cancer cell line	IgG1	42 kDa glycoprotein	Unknown
454A12	SK-BR-3 breast cancer cell line	IgG1	Human transferrin receptor	Iron transport
520C9	Breast cancer membranes	IgG1	c-erbB-2 protooncogene product (heregulin)	Growth/differentiation factor receptor

Six previously characterized murine monoclonal antibodies raised against human breast cancer [3, 5] were chosen for their recognition of distinct tumor-associated antigens
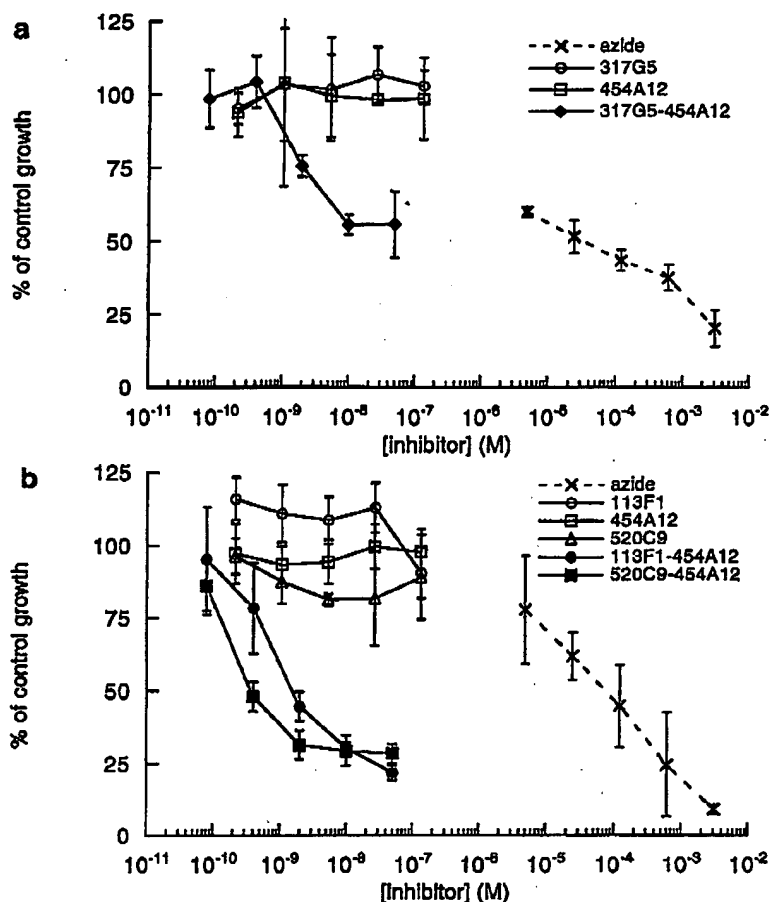
Fig. 1a, b. Effects of antigen forks and unconjugated component antibodies on cell growth. HT-29 cells (a) or SK-BR-3 cells (b) were seeded at 5000 cells/well and serial dilutions of antigen fork heteroconjugates or component antibodies were tested for anti-proliferative effects in 6-day MTT growth assays. Dilutions of sodium azide were included as a cytotoxic positive control. Results are expressed as a percentage of control cell growth in the absence of any antibody or conjugate, and *error bars* indicate 95% confidence intervals for mean growth data

for 4 h at 37° C in 5% CO_2 before the addition of 100 μl solubilization solution. Plates were read for absorbance at 570/630 nm on an enzyme-linked immunosorbent assay plate reader after all blue crystals had dissolved (typically 1–5 days at room temperature in a moist chamber). Error bars on MTT data represent 95% confidence intervals for the means of replicate data.

Results

Cell line binding by component antibodies

Six monoclonal antibodies were selected as potential components for antigen forks (Table 1). These antibodies

recognize a high-molecular-mass mucin, several smaller glycoproteins or glycoprotein complexes, the c-*erb*B-2 protooncogene product, and human transferrin receptor. Three cell lines were chosen for initial testing and two more were added in later experiments. HBL-100 is a non-tumorigenic human mammary epithelial cell line that expresses low to undetectable levels of the antigens recognized by our antibody set. It may be considered as a surrogate for normal cells lacking some tumor-associated antigens and expressing others at low levels. HT-29, SK-BR-3, SK-OV-3 and SW948 are, respectively, colorectal, breast, ovarian and colorectal human cancer cell lines

Table 2. Cell line binding of antigen fork component antibodies

Cell line	mIg	2G3	113F1	260F9	317G5	454A12	520C9
HBL-100	0 (3)	+ (74)	+ (87)	± (34)	0 (5)	+ (77)	± (14)
HT-29	0 (6)	± (16)	± (15)	0 (7)	++ (171)	+ (59)	± (38)
SK-BR-3	± (11)	+ (81)	++ (141)	++ (138)	++ (133)	+ (78)	++ (171)
SK-OV-3	0 (3)	+ (66)	0 (3)	+ (78)	+ (70)	+ (61)	++ (165)
SW948	0 (5)	± (26)	++ (123)	0 (9)	++ (175)	+ (53)	± (38)

Binding of six chosen monoclonal antibodies or normal polyclonal mouse immunoglobulin (mIg) to the HBL-100 human mammary epithelial cell line and four human tumor cell lines was evaluated by flow cytometry as described in Materials and methods. Results are categorized as negative (0; mean fluorescence intensity = 0–10),

weakly positive (±; MFI = 11–50), moderately positive (+; MFI = 51–100) or strongly positive (++; MFI >100). The actual mean fluorescence intensity in channel number is given in parentheses, with 25 channels equal to a doubling in signal intensity
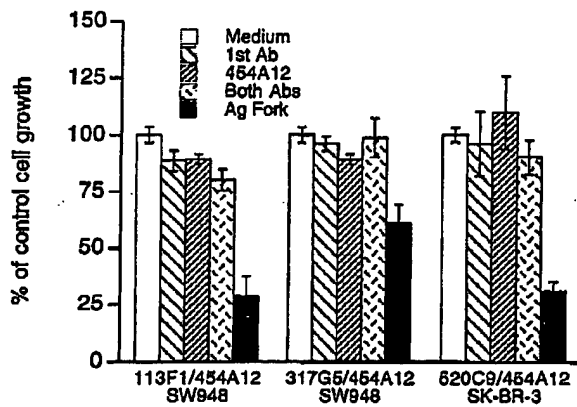
Fig. 2. Effects of antigen forks compared to single or combined component antibodies. Component antibodies, equimolar mixtures of component antibodies, or corresponding antibody heteroconjugates were tested for anti-proliferative effects in 7-day MTT growth assays. The 113F1-454A12 and 317G5-454A12 forks and their components were tested on SW948 colorectal cancer cells seeded at 5000 cells/well, while the 520C9-454A12 fork and its components were tested on SK-BR-3 breast cancer cells seeded at 10000 cells/well. All antibodies were used at 20 µg/ml (133 nM); the 113F1-454A12 fork was used at 10 µg/ml (approximately 25 nM) and the other two forks at 20 µg/ml (approximately 50 nM). Results are expressed as a percentage of control cell growth in the absence of any antibody or conjugate, and *error bars* indicate 95% confidence intervals for the means of growth data

affected growth (69% inhibition). On SW948 cells, component antibody 317G5 alone or combined with 454A12 had no significant effect, and single-component antibodies 113F1 and 454A12 caused marginally significant effects at 12% and 11% inhibition respectively. The combination of 113F1 and 454A12 caused more significant inhibition (20%), but much less than that observed with the 113F1-454A12 fork (71%) or the 317G5-454A12 fork (39%).

Since the three most active antigen forks all contained antibody 454A12 against human transferrin receptor, and since antibodies to transferrin receptor have been reported to inhibit cell growth [33, 35, 36], we compared the three active fork heteroconjugates with a similar SPDP-cross-linked 454A12-454A12 homoconjugate (Table 4). While the 454A12 homoconjugate slightly affected the growth of HBL-100 mammary epithelial cells (7% inhibition; marginally significant at the 95% confidence level), it had no significant effect on the growth of HT-20 or SK-BR-3 cancer cells. As expected from previous experiments, HT-29 cells were significantly affected by 113F1-454A12 and 317G5-454A12 (56% and 46% inhibition, respectively), and SK-BR-3 cells were significantly affected by 520C9-454A12 fork (50% and 68% inhibition in two experiments)

Discussion

We have described a set of "antigen forks" constructed as antibody heteroconjugates. Binding of an antigen fork to a tumor cell is expected to crosslink heterologously two distinct cell surface antigens. Crosslinking of surface markers (as opposed to monovalent binding) frequently affects antigen turnover and/or signal transduction. The effects of an antigen fork may be relatively selective if crosslinking occurs only on cells having both surface antigens, and is not experienced by bystander cells exhibiting only one of the antigens. Furthermore, if the antigens crosslinked have different biological functions, one or both functions may be impaired by the crosslinking, leading to cell death or inhibition of cell growth.

Our antigen forks were generated from a set of mouse monoclonal antibodies recognizing six different human tumor surface antigens. Antibody 2G3 (also called W1) recognizes a high-molecular-mass mucin expressed on carcinoma cells as well as normal mammary epithelium and milk fat globule membranes [17]. Such mucins are

inhibition in 7/7 experiments with SK-BR-3, 2/2 experiments with SW948 and 1/2 experiments with SK-OV-3. The 317G5-454A12 fork caused more than 30% inhibition in 5/6 assays with HT-29 and 2/2 assays with SW948, while the 454A12-520C9 fork gave more than 30% inhibition in 6/7 tests with SK-Br-3 and 1/2 tests with SK-OV-3. Certain other forks showed lower or less consistent levels of activity against the SK-BR-3 cell line, e.g., 2G3-113F1, 2G3-454A12, 113F1-260F9, 113F1-317G5, 113F1-520C9, 260F9-454A12 and 317G5-520C9.

The inhibitory effects of 113F1-454A12, 317G5-454A12 and 520C9-454A12 depended on covalent conjugation of the forks. Figure 2 compares cell growth in the presence of antigen fork heteroconjugates, single-component antibodies, or equimolar mixtures of component antibodies. When 520C9-454A12 and its components were tested on SK-BR-3 cells, only the antigen fork significantly

Table 4. Growth inhibition by antigen forks compared to 454A12 homoconjugate

Conjugate	Mean growth (% control)			
	HBL-100	HT-29	SK-BR-3, Expt. 1	SK-BR-3, Expt. 2
Medium only	100.0±2.4	100.0±3.7	100.0±1.9	100.0±3.5
113F1-454A12	99.1±3.2	44.4±7.9*	97.3±25.4	ND
317G5-454A12	95.3±2.1	54.1±9.5*	104.0±4.5	ND
454A12-454A12	93.2±4.2*	84.2±17.0	97.9±3.0	97.7±4.4
520C9-454A12	104.5±21.9	85.2±1.7*	50.3±4.9*	32.4±1.3*

Cell lines were grown 6 days with or without 10 µg/ml antigen fork heteroconjugate or 454A12-454A12 homoconjugate and growth was measured by MTT assay. Results shown are mean growth ±95% confidence interval of mean from triplicate wells containing antibody conjugate, expressed as a percentage of control growth in wells without conjugate. ND, not determined
* Results where 95% confidence intervals do not overlap with those for control growth

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thought to prevent unwanted cell adhesion and to mediate adhesion via carbohydrate/selectin interactions [9]. While antibodies that bind mucins are in general poorly endocytosed, 2G3 may be exceptional, since its ricin A chain conjugate was moderately cytotoxic to mucin-expressing tumor cell lines [3].

Antibodies 113F1, 260F9 and 317G5 recognize smaller cell-surface glycoproteins found on a wide range of carcinomas and on secretory epithelial structures in a number of normal tissues. The functions of the 113F1 and 260F9 antigens are unknown. Antibody 317G5 binds the pan-epithelial glycoprotein recognized by monoclonal antibodies 17-1A, KS1/4, 323/A3 and GA733; the function of this antigen is also unknown, although sequence homologies to other proteins have been identified [2]. Studies with immunotoxins constructed from 113F1, 260F9 and 317G5 suggest that the antigens recognized by these antibodies are endocytosed at moderate rates [3].

Antibody 454A12 recognizes human transferrin receptor, which has a central role in cellular iron uptake [31]. Transferrin receptor is found at high levels in many tumors, and is also expressed on essentially all rapidly dividing normal cells [16]. It is rapidly and efficiently endocytosed and recycled intact to the cell surface [31, 34].

Finally, antibody 520C9 recognizes an extracellular epitope of the c-erbB-2 (HER2/neu) human protooncogene product [25, 26]. This antigen is the most nearly tumor-restricted in the set, occurring at amplified levels in many breast, colorectal, ovarian and other carcinomas, but on very few normal human tissues [22, 25]; its rate of endocytosis, on the basis of immunotoxin experiments, was moderate [3]. The c-erbB-2 product has been characterized as a growth or differentiation factor receptor involved in signal transduction [10, 18, 19, 23, 38]. Although certain antibodies to c-erbB-2 have been reported to inhibit the growth of cancer cells simply by binding [1, 13, 27–30], we have not observed strong growth inhibition with antibody 520C9.

While some of the above antigens are poorly characterized, and none is perfectly restricted to tumors, they constitute six distinct cell-surface molecules, presumably representing a number of different functions, at least some of which may be sensitive to heterologous crosslinking caused by an antigen fork. When we constructed and tested the 15 possible antigen forks from our set of six antibodies, three forks had particularly strong anti-growth effects against tumor cells. The 113F1-454A12 and 520C9-454A12 forks inhibited SK-BR-3 breast cancer cells, and 113F1-454A12 also inhibited SW948 colorectal cancer cells. The 317G5-454A12 fork was effective against both colorectal cancer cell lines, HT-29 and SW948, but not against SK-BR-3. In each case, one target antigen was strongly expressed on the target cells and the other target antigen (human transferrin receptor) was moderately expressed. Significant growth inhibition was seen at fork concentrations of 0.4–40 μg/ml, or approximately 1–100 nM. Unconjugated component antibodies had little or no effect within the same concentration range. As expected, forks did not affect the growth of the non-tumorigenic HBL-100 mammary line, which expresses low to negligible levels of the relevant antigens.

All three of the most active forks contain an antibody against transferrin receptor, and each also recognizes a cell-surface glycoprotein. Experiments with radiolabeled antibodies and forks indicate that the three highly active forks are endocytosed into their optimal target cells less rapidly than transferrin receptor antibody 454A12, but more rapidly than their other component antibodies (T. Shi, J. Reeder and D. Ring, unpublished data). This suggests that the mechanisms of these three forks may be related either to interference with normal transferrin receptor cycling (and thus with iron transport) or to removal of the other antigens from the cell surface. In the case of 520C9-454A12, the second antigen is the c-erbB-2 growth factor receptor, and its removal may interfere with signal transduction. Although unconjugated antibodies to transferrin receptor have been reported to inhibit the growth of certain cells [33, 35, 36], neither unconjugated 454A12 nor its SPDP-linked homoconjugate was nearly as inhibitory as the 113F1-454A12 and 317G5-454A12 forks to HT-29 cells or the 520C9-454A12 fork to SK-BR-3 cells. The low activity of the 454A12 homoconjugate suggests that the higher activity of the heteroconjugate forks involves their second antigenic specificity, and is not simply a result of increasing the valency or avidity of 454A12 via conjugation.

For the two colorectal cancer cell lines tested, sensitivity to antigen forks correlated with higher levels of antigen expression. The only fork active against HT-29 was 317G5-454A12, and the component antibodies of this fork were the only ones to bind HT-29 (317G5) or moderately (454A12). The two forks active against SW948 were 113F1-454A12 and 317G5-454A12. In this case, 113F1 and 317G5 bound SW948 cells strongly, 454A12 bound moderately, and the other component antibodies bound weakly or not at all.

For SK-BR-3 breast cancer cells, the most consistently active forks were 113F1-454A12 and 520C9-454A12. In both of these combinations, the first component antibody bound SK-BR-3 strongly and the second bound moderately. Interestingly, the 317G5-454A12 fork did not inhibit growth of SK-BR-3 cells despite the facts that (a) 317G5 bound SK-BR-3 strongly and 454A12 bound moderately, and (b) the same fork preparation was active against the HT-29 and SW948 lines, which showed comparable high/moderate levels of component antibody reactivity.

Several forks failed to inhibit growth of cell lines that reacted strongly to moderately with both their component antibodies (e.g. 113F1-317G5 on SW948 cells or 260F9-317G5, 260F9-520C9, 317G5-454A12 and 317G5-520C9 on SK-BR-3 cells). We assume that the 317G5-454A12 preparation contained active bispecific molecules because it inhibited cell lines (HT-29, SW948) that were not inhibited by its component antibodies or by the 454A12 homoconjugate. In other cases where no cell line was strongly inhibited, we cannot rule out the possibility that apparently inactive forks were actually damaged during SPDP conjugation, since we screened only for anti-growth effects and did not separately measure the binding ability of our antibody heteroconjugates after crosslinking.

While we expect antigen forks primarily to crosslink antigens on the same cell, it is possible that a fork could cause cell complexing by binding antigens on different cells. Such complexing might extend to cells that do not

simultaneously express both antigens, and could conceivably affect their growth. We do not believe that cell/cell complexing was a significant factor in the results presented above. The cells used in our assays were adherent, and few cells were in contact in the lightly seeded wells at the time bispecific antibodies were added. Flow-cytometric experiments (data not shown) indicated that our forks at 20 µg/ml had only a limited ability to complex suspended SW948 or SK-BR-3 cells, which did not correlate with their ability to inhibit growth of these cell lines at considerably lower fork concentrations. The highly inhibitory 317G5-454A12 and 520C9-454A12 forks failed to complex SW948 cells and SK-BR-3 cells respectively. The highly inhibitory 113F1-454A12 fork did complex SW948 cells, but so did the inactive 113F1-317G5 fork, to a similar extent.

In vivo, we believe that heterologous crosslinking of antigens on the same cell will be more important than bridging of antigens on different cells. Cell/cell bridging would be limited to contact zones of different solid-tissue cell types, or to interactions of circulating blood cells with solid tissues. In many situations, steric and kinetic factors may disfavor binding of an antibody molecule to two cells rather than two antigens on the same cell. Nevertheless, the possibility of cell bridging deserves continued attention, since it could undermine any enhanced tumor selectivity of antigen forks, and might result in effects on normal tissues that express only one antigen.

While several of the forks we screened showed strong effects on tumor cell proliferation, none completely inhibited growth or killed all tumor cells at the concentrations used, although greater inhibition was observed in longer MTT assay periods. In subsequent experiments, we have observed that combinations of antigen forks with small-molecule chemotherapeutic drugs, including cisplatin and the iron chelater deferoxamine, provide synergistic and complete killing of target tumor cells (Sylvia T. Hsieh-Ma, unpublished data). Furthermore, monovalent antigen forks constructed by linking Fab' fragments instead of whole antibodies retain activity (Tim Shi and Sylvia T. Hsieh-Ma, unpublished data), which has led us to begin making fork-secreting hybrid hybridomas.

The three active antigen forks identified in this paper may be interesting as prototypes of cancer therapeutic agents, since their tumor selectivity is likely to be greater than that of their component antibodies. The 520C9-454A12 fork is particularly attractive, since the c-erbB-2 antigen recognized by 520C9 already has a very restricted normal tissue distribution [22, 25], which does not overlap with the normal tissue structures that express high levels of transferrin receptor [6, 11]. Antibodies 113F1 and 317G5 bind considerably more normal tissue structures than 520C9 (unpublished data), but again, there is little overlap between the secretory epithelial structures recognized by these antibodies and sites of high transferrin receptor expression.

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Cancer Immunol. Immunother. (1994), 39(1), 41-8

Proceedings of the American Association for Cancer Research Annual Meeting, (1994) Vol. 35, No. 0, pp. 219

Thanks,

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1305

A Phase I clinical trial in cancer patients with a new monoclonal antibody (MAb), FC-2.15. Mordoh J., Silva C., Albarellos M. & Kairiyama C. Instituto de Investigaciones Bioquimicas "Fundacion Campomar" and Instituto Alexander Fleming, Buenos Aires, Argentina.

FC-2.15 is a new murine IgM MAb that recognizes previously undescribed antigens present in proliferating breast cancer cells and normal peripheral granulocytes. A Phase I clinical trial was performed in 10 patients with advanced cancer (breast=4, colon=1, melanoma=2, lung= 1, medullary thyroid=1, skin epidermoid carcinoma=1). FC-2.15 was administered by i.v. infusion every other day ; nine patients received 4 infusions and one patient 3 infusions. One patient received 2 cycles of treatment. Total doses of FC-2.15 ranged between 2.4 and 5.1 mg/kg. Maximal FC-2.15 serum concentration ranged between 2.2 µg/ml and 5.5 µg/ml, and its serum half-life (t1/2) was about 9 hr. All patients developed HAMA. The most consistent side effect (10/10 patients) was a profound and selective neutropenia wich occured within 1 hr after the start of each infusion and reversed within 1 hr after its discontinuation . Other frequent side effects included fever < 38 ℃ and chills that were easily manageable. None of these effects required dose reduction or treatment interruption ; the patient which received two treatment cycles did not develop allergic reactions. A complete response of a basocellular carcinoma and a sustained (6 months) >50 % partial response of breast carcinoma liver metastases were observed.

1306

Radioimmunodetection of epithelial cancers with an anti-MUC1 murine monoclonal antibody. Sharkey, R.M., Dion, A.S., Swayne, L.C., Conte, P., Markowitz, A., and Goldenberg, D.M. Garden State Cancer Center at the Center for Molecular Medicine and Immunology, and St. Michael's Newark, NJ 07103, and Morristown Memorial Hospital, Morristown, NJ 07950.

We have investigated an IgG$_1$ murine monoclonal antibody (MAb) that is reactive with a tandemly repeated peptide epitope of MUC1. Immunohistology indicated that the MAb reacted with 84% (21/25) and 93% (13/14) of primary and metastatic breast cancers, respectively. Also, significantly elevated serum levels of MUC1 were found for at least 50% of breast (all stages), colon, lung, prostate, ovarian, and pancreatic cancer patients. Clinical trials have been initiated to determine the targeting ability of this antibody. ^{131}I-labeled IgG or F(ab')$_2$, as well as ^{99m}Tc-Fab' fragments, have been studied in 5 breast, 3 ovarian and 1 lung cancer patient. Tumors were localized in all but 1 of the breast cancer patients. In 2 patients, disease was first seen by radioantibody imaging with subsequent confirmation by magnetic resonance imaging. Although MUC1 is found in the plasma, < 25% of radiolabeled antibody in the plasma was complexed after injection in only 3/6 assessible patients. These studies suggest that this anti-MUC1 MAb may be a useful antibody for detection or therapy of epithelial cancers. (Supported in part by PHS grant CA39841.)

1307

Initial clinical investigations of PAM4, a new murine monoclonal antibody against pancreatic cancer. Sharkey, R.M., Gold, D.V., Markowitz, A., Swayne, L.C., Conte, P., and Goldenberg, D.M. Garden State Cancer Center at the Center for Molecular Medicine and Immunology and St. Michael's Hospital, Newark, NJ 07103, and Morristown Memorial Hospital, Morristown, NJ 07950.

A new murine monoclonal antibody designated PAM4 was generated against a mucin from pancreatic cancer. By immunohistology, PAM4 showed reactivity with greater than 80% of pancreatic cancers and yet was unreactive with normal adult pancreatic tissue. Animal studies showed excellent targeting with ^{131}I-labeled PAM4 (21 to 44 percent of injected dose per gram of tumor at day 3 post injection for 4 different human pancreatic tumor lines). Five patients with proven pancreatic cancer have been studied using ^{131}I-PAM4 IgG or ^{99m}Tc-PAM4 Fab'. Definitive tumor localization was seen in 4/5 patients. Dosimetry from 2 patients receiving ^{131}I-PAM4 IgG predicted tumors received 10-20 cGy/mCi with tumor/red marrow dose ratios ranging from 3-10. These results suggest that PAM4 is a promising antibody for targeting pancreatic cancer. (Supported in part by PHS grants CA39841 and CA54425 and a grant from the Robert Leet and Clara Guthrie Patterson Trust.)

1308

Clinical evaluation of Tc-99m labelled anti-TF MAb 170 in the assessment of metastatic colorectal cancer. Kirsh, J., Smith A., Reilly R., Gallinger S., & Stern H. Departments of Surgery and Nuclear Medicine, Mount Sinai Hospital and The Toronto Hospital, University of Toronto, Toronto, Ontario M5G 1X5.

MAb is an antibody directed against a membrane protein associated with the Thomsen-Friedenreich antigen, expressed on the majority of human adenocarcinomas. Our objective was to evaluate the efficacy of Tc-99m labelled MAb 170 for assessment of metastatic colorectal cancer (MCRC). Twenty patients (6 with concomitant primary CRC) were injected i.v. with 1800 MBq (2 mg) of Tc-99m MAb 170. At 22 h p.i. planar and SPECT images of the abdomen and pelvis were obtained. Sixteen patients (pts) underwent laparotomy within 48 h p.i. and samples of tumor and normal tissues were obtained. Radioactivity in these tissues (%ID/g) was measured by scintillation counting. MAb 170 correctly identified the primary tumor in 3/6 pts and MCRC in 3/5 pts with biopsy confirmation. Two FN studies were in 1 pt with liver metastases and 1 pt with a pelvic nodule. Two additional pts with positive MAb 170 scans await surgical follow-up. CT or US identified MCRC in 4/5 pts. Two pts with negative MAb 170 scans who underwent laparotomy after radiological tests suggested MCRC were found to have non-malignant disease. Two pts with high, rising CEA exhibited positive MAb 170 scans but MCRC could not be identified at laparotomy (FP). Radioactivity in the tumor or normal bowel was 0.00004-0.005%ID/g and 0.00001-0.005%ID/g respectively. We conclude that Tc-99m MAb 170 may have an important role in the assessment of occult MCRC.

1309

Phase I trial of a bispecific murine monoclonal antibody targeting c-erbB-2 and CD16. Weiner, L.M., Ring, D.[1], Li, W., Palazzo, I.E., Davey, M., Rivera, V., Alpaugh, R.K. Fox Chase Cancer Center, Philadelphia, PA 19111, [1]Chiron Corp., Emeryville, CA 94608.

Bispecific monoclonal antibodies (BsMAb) can direct tumor lysis by effector cells via defined cytotoxic trigger molecules. The BsMAb 2B1 promotes c-erbB-2 tumor cell lysis by human NK cells and macrophages expressing CD16 (i.e., FcγRIII), and is effective in murine xenograft models. Neutrophils (PMN) express an isoform of CD16 that does not trigger lysis. In a dose-escalating Phase I clinical trial, nine patients with c-erbB-2(+) tumors have been treated i.v. with 1 hr BsMAb infusions on days 1,4,5,6,7 and 8, at 1 mg/m^2 (n=3) or 2.5 mg/m^2 (n=6) 2B1 per dose. The MTD has not been reached. Treatment causes fevers, rigors, reversible neutropenia and loss of circulating monocytes and NK cells. Treatment also alters the distribution of ^{111}In-labeled autologous leukocytes, with tumor localization noted in at least one patient. Circulating 2B1 retains its dual binding characteristics. Peak levels of 240-2260 ng/ml murine IgG have been detected, with binding to circulating and peritoneal PMN, NK cells and mononuclear phagocytes. One clinical response has been observed in a patient with chest wall recurrence of breast cancer. The binding of this BsMAb to CD16-expressing leukocytes has potent biological effects which may be exploited at higher, tumor-binding 2B1 doses.

1310

Induction of complete remission in advanced refractory chronic lymphocytic leukemia (B-CLL) by CAMPATH-1H with disappearance of malignant trisomy-12 clone determined by fluorescence in situ hybridization (FISH). H. Ozer, A. Steagall, C. Coppedge, M.A. Collier and J. Purvis. University of North Carolina, Chapel Hill, NC 27599 and Burroughs Wellcome Co., RTP, NC 27709.

CAMPATH-1H is under investigation in the therapy of non-Hodgkin's lymphoma and B-CLL. CAMPATH 1-H, a "humanized" version of CAMPATH-1G (secreting IgG2b) is produced by introducing hypervariable regions from the heavy and light chain variable domains of the rodent antibody (Ab) into a human IgG-1 framework. The Ab is directed against CDw52 (CAMPATH-1) antigen which is present on at least 95% of all human peripheral blood B and T lymphocytes and can selectively lyse lymphocytes in the presence of complement. Patient 67 was a 47 year old white male diagnosed in 1984, pretreated for 8 years with pentostatin/interferon, chlorambucil, splenic irradiation, and fludarabine, refractory to each regimen. In 9/92 he developed splenomegaly ≥ 14 cm. WBC > 600,000 and a 4 cm right upper lung mass. He received CAMPATH-1H beginning at 25 mg/m2 escalated to 75 mg/m2 tiw IV for 3 months per protocol BW-01. He achieved a complete remission (CR) at 4 weeks and has remained in unmaintained CR for 12 months. Karyotyping in 9/92 confirmed presence of a trisomy 12-positive clone with a normal 46 XY karyotype in 2/93. Absence of minimal residual disease by FISH was documented in 2/93. These data confirm the potential for major clinical and cytogenetic responses in advanced refractory CLL to CAMPATH-1H.

2050

Cytotoxicity of Conjugates Between LL2 and Derivatives of *Pseudomonas* Exotoxin Toward B-Cell Non-Hodgkin's Lymphoma. Kreitman, R.J., FitzGerald, D.J., Hansen, H., Goldenberg, D.M., and Pastan, I. Lab. of Molecular Biology, DCBDC, NCI, NIH, Bethesda, MD 20892; Immunomedics Inc., Warren, NJ 07059; Center for Molecular Medicine and Immunology, Newark, NJ 07103.

LL2 is a pan-B-cell monoclonal antibody which in radioiodinated form has induced remissions in patients with lymphoma. We have chemically coupled LL2 to PE38KDEL, a derivative of *Pseudomonas* exotoxin (PE) which does not bind to the PE receptor. PE38KDEL is devoid of amino acids 1-252 and 365-380 of PE, and contains a -KDEL mutation instead of -REDLK at the carboxyl terminus. LL2-PE38KDEL was cytotoxic toward several Burkitt's lymphoma lines, with an ID_{50} of 2 ng/ml on Daudi cells and about 6 ng/ml on Raji, CA-46 and JD-38 cells. LL2 alone had no cytotoxicity toward the malignant cells and excess LL2 could prevent the cytotoxicity of LL2-PE38KDEL, indicating the immunotoxin and LL2 bind to the same antigen. LL2-PE38KDEL prevented the growth of CA-46 cells in nude mice. LL2-Fab'-PE38KDEL was produced by chemically coupling the Fab' fragment of LL2 to PE38KDEL. LL2-Fab'-PE38KDEL was also cytotoxic, with an ID_{50} of 2 ng/ml toward CA-46 cells. LL2-Fab'-PE38KDEL was given to nude mice with visible CA-46 xenografts and showed significant antitumor activity. Both LL2-PE38KDEL and LL2-Fab'-PE38KDEL are being investigated as potential therapeutic agents for non-Hodgkin's lymphoma. Supported in part by NIH grant CA39841 CDMG.

2051

TUMOR NECROSIS TREATMENT OF HUMAN CANCERS. Epstein, A.L., Khawli, L.A., Strum, S.[*], Thompson, L.[*] and Taylor, C.R. Department of Pathology, University of Southern California School of Medicine, Los Angeles, CA 90033 and [*]Brotman Medical Center, Culver City, CA. 90231.

A new method of cancer imaging and therapy has been developed which utilizes necrotic cells as the target for the selective binding of monoclonal antibodies (MAbs) to human tumors. Designated *Tumor Necrosis Treatment (TNT)*, this novel approach is applicable to a broad spectrum of human cancers and circumvents many of the limitations of MAb therapy directed against tumor cell surface antigens. Initial imaging trials in man utilizing 2-5 mCi I-131 labeled TNT-1 F(ab')2 demonstrated positive images in 5/7 cases at sites of known primary and secondary lesions including liver metastases in two patients and lymph nodes in one. Whole body and region of interest scans showed no evidence of MAb accumulation in normal organs. More recently, an IND sponsored therapeutic protocol which included weekly x 3 doses of biotinylated TNT-1 MAb followed 4 days later by 20 mCi of I-131 labeled streptavidin was begun for advanced prostate cancer patients. Early results indicate several significant findings including (1) rapid uptake of radiolabeled streptavidin in bone metastases first seen at 5 hours, (2) evidence of tumor destruction determined by rising prostate specific antigen (PSA) early after therapy (tumor lysis) followed by a decreased PSA value, (3) decreased bone pain, and (4) no evidence of toxicity. Finally, dehalogenation of the radiolabeled streptavidin was not observed. These early findings indicate that TNT is a promising new approach for the imaging and therapy of solid tumors such as prostate carcinoma.

2052

Radioimmunotherapy of Relapsed B-Cell Lymphoma with [90]Yttrium Anti-Idiotype Monoclonal Antibodies. Royston, I., Parker, B.A.[*], White, C.A.[+], Miller, R.A.[**], Hupf, H.[***], Starr, R., Shawler, D.L., and Halpern, S.E. , San Diego Regional Cancer Center, San Diego, CA 92121, [*]UCSD, La Jolla, CA 92093, [**]Idec Pharmaceutical Corp., Mountain View, CA 94043, and [***]Hybritech, Inc., San Diego, CA 92121.

We initiated a phase I/II trial of [90]Yttrium (Y)-conjugated anti-idiotype (anti-Id) monoclonal antibody therapy to evaluate its toxicity and efficacy in relapsed B-cell non-Hodgkin's lymphoma. Six patients received ^{90}Y-anti-Id with cumulative doses of 10-54 mCi ^{90}Y and 1000-4550 mg unlabeled anti-Id. Tumor imaging with ^{111}In anti-Id was demonstrated prior to ^{90}Y infusion for each patient. Bone marrow toxicities were nadir WBC 1800-4600, nadir granulocytes 680-3270, and nadir platelets 34,000-178,000. Non-hematopoietic toxicities were minimal. No patients developed human anti-mouse antibodies. Dosimetry calculations suggest tumors received a dose of 18-43 cGy/mCi [90]Y. One patient had a complete response, 2 had partial responses, 2 had stable disease, and 1 had progressive disease. Response duration was 2-12 months. The complete response relapsed at a new site with Id disease after 12 months. The attainment of free circulating mouse IgG in the presence of low circulating Id appeared important for tumor targeting and clinical benefit. The observation of anti-tumor responses in a heavily pretreated population warrants further study of repetitive, low dose ^{90}Y-anti-Id therapy.

2053

Monoclonal antibody distribution in tumors: Theoretical and experimental validation of the "binding site barrier" hypothesis. Weinstein, J.N.[*], Juweid, M.[**], van Osdol, W.[*], Sato, J.[*‡], Fujimori, K.[*], Saga, T.[**], Heya, T.[*‡], and Neumann, R.[**] [*]National Cancer Institute, [**]Clinical Center, NIH, Bethesda, MD 20815, U.S.A.; [‡]Takeda, Inc., Osaka, Japan.

We recently developed new quantitative models for the penetration of monoclonal antibodies (MAb) and MAb radioconjugates into microscopic nodules representing either primary or metastatic solid tumor (Canc. Res. 51: 4776-4784, 4821-4827, 1991). These models refine the "binding site barrier" hypothesis, the idea that MAbs can be prevented from penetrating tissues or tumors by the very fact of their successful binding to antigen (Annals N.Y. Acad. Sci. 507: 199, 1987); they also highlight the consequences of non-uniform distribution for radiation microdosimetry. Previous experimental studies have shown heterogeneous antibody distribution in tumors, but there has been no direct experimental proof of the "binding site barrier" hypothesis. Using the new quantitative models to guide design of multiple-label experiments, we have now obtained such proof in Line 10 bile duct carcinoma of guinea pigs -- by using a combination of double-label autoradiography and double-label immunohistochemistry to determine simultaneously the histological location in tumor of (i) i.v.-administered MAb; (ii) co-administered isotype-matched control MAb; (iii) antigen; (iv) blood vessels. At low MAb dose, binding severely hindered penetration into antigenic patches as small as 300 μm; the barrier could be overcome by raising the dose but at a cost in specificity ratio. The same general principles of micropharmacology are expected to apply to biological ligands other than antibodies, including those secreted by genetically modified cells.

2054

Effective targeting of human lymphocytes and macrophages to tumor by a bispecific monoclonal antibody (BsMAb) binding human c-erbB-2 and FcγRIII. Lee,G., Weiner,L., Ring,D.[1], Holmes,M., Alpaugh,K., Garcia de Palazzo,I. Fox Chase Cancer Center, Philadelphia, PA 19111 and [1]Cetus Corporation, Emeryville, CA 94608.

BsMAbs which bind to tumor and effector cells can trigger cytotoxicity even when effector cell Fcγ receptors (FcγR) are occupied by competing immunoglobulin. The BsMAb 2B1 is produced by a quadroma derived by fusion of hybridomas 520C9 (anti-c-erbB-2) and 3G8 (anti-FcγRIII). Highly purified 2B1 is a potent promoter of *in vitro* lysis of c-erbB-2-expressing tumors by human large granular lymphocytes (LGLs) and macrophages expressing FcγRIII. Prior cellular activation by cytokines is not required for substantial lysis, which is unimpeded in human serum or whole blood. 2B1 recognizes nearly all tumors known to overexpress c-erbB-2 protein despite its monovalent antigen binding properties. Treatment of *scid* mice bearing SK-OV-3 human tumor xenografts with 2B1 results in significant prolongation of survival and some cures. 2B1 meets the criteria for clinical development to test the hypothesis that BsMAbs can promote *in vivo* accumulation of cytotoxic effectors in tumor.

2055

Selective photodynamic killing of human B lymphoma cells using monoclonal antibody-porphyrin conjugates.
W.C. Biddle, J.D. Pancook, D.A. Musser, A. Oseroff.
Life Technologies Inc., Grand Island, N.Y. and Dept. of Dermatology, Roswell Park Cancer Institute, Buffalo, N.Y.

A model system for the selective killing of B cell malignancies utilizing the photosensitizer 5,10,15,20-tetracarboxyphenylporphine (TCPP), covalently coupled to the murine monoclonal antibody (MAb) Lym-1, was expanded to include several other B cell-reactive MAb's. The B1, B3, B4 and J5 MAb's bind the CD20, CD22, CD19, and CD10 antigens respectively. A water soluble carbodiimide and N-hydroxysuccinimide were used to covalently attach TCPP to immunoglobulin molecules, which retained binding specificity and exhibited photodynamic activity subsequent to conjugation. Upon light activation, the conjugates showed selective *in vitro* killing of antigen-positive cells, characterized by severe membrane damage. Cytotoxicity was studied using the conjugates individually and in combination. Results indicated that these photoactive immunoconjugates may have utility for *ex vivo* purging of bone marrow for autologous transplantation procedures.

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PHASE I TRIAL OF 2B1, A BISPECIFIC MURINE MONOCLONAL ANTIBODY TARGETING c-erbB-2 AND CD16. L.M. Weiner, D. Ring[1] W. Li, I.E. Palazzo, M. Davey, V. Rivera, R.K. Alpaugh, Fox Chase Cancer Center, Philadelphia, PA 19111 and [1]Chiron Corp., Emeryville, CA 94608.

Bispecific monoclonal antibodies (BsMAb) can direct tumor lysis by effector cells via defined cytotoxic trigger molecules without competition by host IgG. The BsMAb 2B1 promotes c-erbB-2 tumor cell lysis by human NK cells and macrophages expressing CD16 (i.e., FcγRIII), and is effective in a murine xenograft model. Neutrophils (PMN) abundantly express an isoform of CD16 that does not trigger tumor lysis. In a dose-escalating Phase I clinical trial, eleven patients with c-erbB-2(+) tumors and ECOG P.S. = 0 or 1 have been treated i.v. with 1 hr BsMAb infusions on days 1,4,5,6,7 and 8, at 1 mg/m^2 (n=3), 2.5 mg/m^2 (n=6) or 5.0 mg/m^2 (n=2) 2B1 per dose. The MTD has not been reached. The severity of fevers, rigors, transient Grade 4 neutropenia and the loss of circulating monocytes and NK cells are not dose-dependent. Peak serum levels of TNF (2400 pg/ml), IL-6 (9000 pg/ml), IL-8 (11 pg/ml) and elastase (600 ng/ml) are found 1-4h following the start of BsMAb infusion. Tumor localization of ^{111}In-labeled autologous leukocytes has been noted. Dose-related peak serum levels of 240-2260 ng/ml murine IgG have been detected, as has HAMA. Circulating 2B1 retains its dual binding characteristics and can be found on circulating and peritoneal PMN, NK cells and mononuclear phagocytes by flow cytometry. Treatment enhances peripheral blood NK activity and LAK precursors, resulting in augmented in vitro growth inhibition of c-erbB-2(+) SK-OV-3 cells. A clinical response was observed in a patient with an evaluable chest wall recurrence of breast cancer. This first clinical trial of a BsMAb targeting tumor and CD16 has demonstrated potent biological effects which may be exploited at higher, tumor-binding 2B1 doses. The role of PMN in promoting these effects requires examination.

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SELECTIVE T-CELL (CD5/CD8) DEPLETION OF BONE MARROW UTILIZING POLYSTYRENE DEVICES. L. YU. Department of Pediatrics, LSU Medical Center and Children's Hospital, New Orleans, LA

In various animal model systems, it has been demonstrated that graft vs. host disease (GVHD) is mediated by the mature T-cells present in the donor marrow inoculum. We report here the use of a simple system in which T-cells from bone marrow (BM) are depleted through passage on the T25 microcelletor cell culture flask (Applied Immune Sciences Inc., Menlo Park, CA) a solid phase polystyrene device to which monoclonal antibodies to CD5, CD8, and SBA have been covalently bound yielding an SBA$^-$ (5/8)$^-$ cell fraction. In this procedure, BM mononuclear cells (BMMC) obtained from 15 consecutive malignant patients undergoing follow-up BM exams, were incubated with T25 SBA flasks for 1 hour. The non-adherent cell suspension (SBA$^-$) was then incubated with the T25 CD5/CD8 flasks for another hour. Samples of marrow at successive stages of processing were analyzed by immunofluorescence using flow cytometry. Recovery of non-adherent SBA$^-$ (5/8)$^-$ cells was 74% after double passage over AIS Cellector system. The mean log T-cell depletion was 2.3 (range 2-3). The use of T25 microcellector - T-cell has the advantages of comparable, if not better cell recovery, ease of operation and manipulation in a closed system. Clinical trials will determine the relevance of these results on engraftment, GVHD, and immune reconstitution.

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PROTEIN A IMMUNOADSORPTION TREATMENT OF HIGHLY REFRACTORY THROMBOCYTOPENIC PURPURA (ITP). R. Anderson, J. H. Saiers, D. A. Clark, D. R. McKinney. Veterans Affairs Medical Center, Albuquerque, NM 87108

Chronic ITP frequently complicates lymphoid malignancy and dysregulation and may be life threatening. We report a case of a 70 year old man who had recurrent bleeding from lupus-associated ITP refractory to splenectomy and immunomodulation with prednisone, cyclophosphamide, vincristine, vinblastine, azathioprine, alpha-interferon, danazol and cyclosporin, as well as exhaustive plasmapheresis. For two years after failure of these treatments he received 220gms per month maintenance intravenous immune globulin (IVIG) to maintain an average platelet count of 35x10^9/L (range 8-75x10^9/L). He was then treated with extra-corporeal immunoadsorption of 2000ml plasma on staphylococcal protein-A silica columns (Prosorba) every other day for six treatments. Four weeks after this treatment his platelet count stabilized at an average of 29x10^9/L for ten weeks. The patient received no IVIG therapy during this period. Since, he has required IVIG only for exacerbations of thrombocytopenia and bleeding with intercurrent viral infection in maintaining an average platelet count of 26x10^9/L. Prior to immunoadsorption the cost of IVIG averaged $4500/month. The cost of IVIG therapy since immunoadsorption has decreased by greater than 75%. This case emphasizes the efficacy of this treatment modality in extraordinarily refractory ITP as well as its potential cost-effectiveness.

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ORAL PENTOXIFYLLINE AS INHIBITION OF THE CYTOKINE SYNDROME INDUCED BY ANTI-CD3 STIMULATION. O. Wesly, J. Hank, M. Albertini, J. Schiller, J. Gan, K. Oettel, A. Borchert, K. Moore, A.M. Bastin, R. Bechhofer, B. Storer, C. Gambacorti, and P.M. Sondel. University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792

In vivo T lymphocyte stimulation with anti-CD3 monoclonal antibodies (OKT3), induces a cytokine syndrome which results from production of large quantities of TNFα, IL-2 and IL-6 by the stimulated lymphocyte population. We have conducted a Phase I clinical trial of escalating doses of anti-CD3 monoclonal antibodies (from 6 μg/m^2 to 3000 μg/m^2) with continuous infusion IL-2 at 3x10^6 U/M^2 as immune stimulation in patients with advanced malignancies. At the 600 and 3000 μg/m^2 anti-CD3 dose levels patients experienced marked toxicity following antibody infusion characterized by hypotension, fever, vasodilation and elevations in serum BUN, creatinine and bilirubin. Those patients developing marked symptomatology also showed markedly elevated TNFα levels in serum one hour post anti-CD3 administration. In an effort to abrogate the clinical toxicity associated with high levels of TNFα, a group of 4 patients was treated at the 600 μg/m^2 anti-CD3 dose level with oral Pentoxifylline (PTX) (at 2 grams daily in 5 divided doses) before, during and after anti-CD3 dosing. PTX is a methylxanthine shown in vitro to be capable of inhibiting TNFα production following anti-CD3 stimulation. Patients in the PTX group appeared clinically more stable after anti-CD3 treatment than those patients previously treated with similar doses of anti-CD3 but without PTX. The PTX group showed smaller elevations and smaller percentage changes from baseline in BUN, Creatinine, and Bilirubin levels as well as more stability in blood pressure determinations. However, statistical significance for the differences between the PTX treated and non treated groups was not demonstrated possibly reflecting the low numbers of patients treated in the PTX group. Only one of four patients on the anti-CD3+PTX regimen was observed to have marked symptomatology following anti-CD3 administration, and GI upset may have impeded this patient from receiving all of the planned PTX dose. TNFα levels were found not to be lower in the PTX-treated as compared to the PTX-untreated group. Thus our results do not prove a correlation between improved clinical outcome and lower TNFα levels. Nonetheless as others have documented, PTX can ameliorate some clinical toxicities of cytokine treatment. This limited clinical experience indicates PTX may be useful in ameliorating toxicities due to the cytokine syndrome following anti-CD3 administration.

PAC261.A1 A46

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Thanks,

Jennifer Hunt
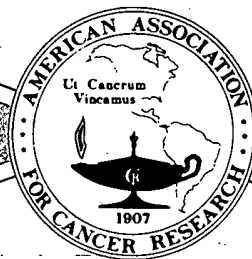Patent Examiner, Art Unit 1642
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Eighty–Third Annual Meeting
of the American Association
for Cancer Research

May 20–23, 1992

San Diego, California

Volume 33

March 1992